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PRESERVATION OF DAMP GRAIN WITH PROPIONIC ACID

by

(C) MARIA DANUTA ULRICH
B.Sc.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

PRESERVATION OF DAMP GRAIN WITH PROPIONIC ACID

submitted by Maria Danuta Ulrich, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor

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Date ..April 25, 1974.....

ABSTRACT

A mixture of acetic and propionic acids was found to be effective in the preservation of stored moist grain.

Examination of supernatants of treated grain solutions for microbial growth is proposed as a reliable test of preservative action.

Dose-response curves showed that acetic and propionic acids were equivalent in killing grain microorganisms. The viable count of survivors decreased geometrically. Similar results were obtained when grain was treated with the sodium salts of the acids. The salts, however, were not as effective as the acids.

Several approaches were used to determine whether pH was entirely responsible for the killing of grain microorganisms. Evidence is presented indicating that the biocidal effect is due to the concentration of undissociated acid.

Comparison of total viable counts of survivors after exposing grain to various concentrations of acetic and propionic acids individually, and in a 1:1 proportion, revealed equivalent killing effects.

Biostatic concentrations of acid are biocidal for the grain microflora when exposure to acid is prolonged. For biocidal concentrations, the majority of killing occurs within one minute upon the addition of acid.

Viable count determinations showed that grains of extreme surface pH values had a reduced grain microflora.

Experiments were undertaken to determine the ability of the

grain microflora to utilize the acids employing radiorespirometry with ^{14}C - carboxyl labelled acids. The grain microflora metabolized both acetic and propionic acids when in dilute solutions and the former more readily than the latter, both in rate and extent. No propionic acid and only a minimal amount of acetic acid breakdown was observed at a higher concentration of these acids. Significant reduction in initial viable count of inoculum suggested that microorganisms capable of acid utilization were killed. That grain absorbs acid was indicated by the detection of radioactivity in ground grain kernels as well as a decrease in the amount of $^{14}CO_2$ released when grain was present in reaction mixtures.

Exposure of grain microorganisms to increasing concentrations of acid selected for molds and yeasts, with sporing and capsulated microorganisms exhibiting the greatest resistance to acid sterilization.

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ABBREVIATIONS

cpm	- counts per minute
cm	- centimeter
DMPOP	- 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene
dpm	- disintegrations per minute
g	- gram
hr	- hour
l	- liter
M	- molar
mc	- millicurie
mg	- milligram
ml	- milliliter
mm	- millimeter
mM	- millimolar
mmol	- millimole
N	- normal
nmol	- nanomole
PCA	- plate count agar
POPOP	- 1,4-bis-[2-(5-phenyloxazolyl)]-benzene
PPO	- 2,5-diphenyloxazole
psi	- pounds per square inch
rpm	- revolutions per minute
µc	- microcurie

µg	- microgram
µl	- microlitre
µmol	- micromole
v/v	- volume per volume
w/v	- weight per volume

INTRODUCTION

There are many advantages in the preservation of moist grain. Recently, Chemcell Limited has undertaken this project in Alberta with rather promising results. Since preservation dealt with the elimination of microbial growth on grain, it was felt that the problem should be examined from a microbiological point of view.

Like any novel field of research, before questions can be answered difficulties in methodology must be overcome first. In addition, the investigation had to be conducted in such a manner as to satisfy the needs of the company distributing the preservative. Following are the main issues that were to be resolved:

1. Since the grain preservative consists of acetic and propionic acids, one of the primary objectives was to establish their individual roles in preservation in comparison to their combined effects.
2. The preservative acids are volatile and corrosive weak organic acids. Could they be replaced by their milder salts for ease of handling? This entails demonstrating that the salts themselves, or in combination with acid are equivalent in action to the acids.
3. Another aim was to ascertain how the duration of exposure influenced preservation.
4. Due to the bulk of grain being treated, a simple but reliable means of determining the effectiveness of the preservative acids was required.

5. A problem of perhaps more interest to the microbiologist was to investigate whether pH alone was entirely responsible for biocidal activity.

6. The final objective involved the ability of the grain microflora to utilize the preservative acids.

LITERATURE REVIEW

I. The Uses of Propionates as Preservatives

It has been shown by the World Health Organization that approximately 20 per cent of the world's food supply is lost as a result of spoilage by microorganisms. With the ever increasing world population, food shortage is becoming inevitable. Thus the use of preservatives in food has gained wider application with some hope of partially alleviating this problem. In order for a chemical to be used as a food preservative, it should meet certain requirements such as those proposed by the Codex Alimentarius Commission of the Food and Agriculture Organization and the World Health Organization (Kermode, 1972):

1. The nutritional value of the food must be maintained.
2. The food must be palatable after treatment.
3. The suggested level of use must not be toxic to the consumer, nor should it exceed that amount as to reasonably achieve the desired effect. These levels are set by government agencies.
4. The chemical should conform to approved standards of purity, as well as be subject to toxicological evaluation and observations for any possible detrimental effects.
5. It should also be taken into consideration that certain additives be approved for use in certain foods for specific purposes and under specific conditions.

Since they satisfy these principles, the organic preservatives, propionic acid and its sodium and calcium salts have played major roles in arresting microbial spoilage of food products since the early 40's and are still in extensive use today. It is of interest to note that propionic acid is found in nature in appreciable concentrations, and occurs naturally in many foods.

The first attempt in using propionates as preservatives was undertaken by the baking industry to curb the condition known as "rope" in bread, which is generated by a bacterium, Bacillus mesentericus. After baking, the bread is also subject to contamination by molds, especially those of the Aspergillus and Penicillium genera. The economic significance of forestalling mold contamination is appreciated when the overwhelming rate of dispersion of mold spores is taken into account.

One of the earliest quantitative studies in determining the effectiveness of calcium propionate in controlling the rope condition was carried out by O'Leary and Kralovec (1941). Since calcium propionate is a solid, for easier handling it is often used in preference to liquid propionic acid. When the dough was mixed with 0.188% calcium propionate and inoculated with $4x10^5$ bacteria per loaf, the bread had not developed "rope" after 12 days in contrast to the uninoculated control which had after 2 days. On the other hand, Ingram, Ottaway and Coppock (1956) found that bread with 0.35% calcium propionate showed no evidence of "rope" after an interval of more than 21 days, whereas 0.18% did as early as 3 to 6 days. In these experiments the dough was not inoculated with rope-producing bacteria, but the baked loaves were stored under conditions which enhanced "rope".

Lowering the pH by the addition of propionic acid also inhibits "rope": less acid is required than the salt but there is a disadvantage as not only bacteria but yeasts are also killed. Addition of more yeast can curb this problem. Nevertheless, the low pH causes the bread to become unpalatable and its physical characteristics are often unappealing.

At present, either calcium propionate or sodium propionate (MYCOBAN) are used as bread preservatives and the latter more extensively in other baking products with an equal amount of salt being omitted. In the United Kingdom a maximum concentration of 0.3% calculated on the weight of flour is permitted as compared to 0.2% in Canada.

The use of propionates and propionic acid as preservatives has been extended to agricultural products. As early as 1945, Wolford and Andersen demonstrated that sodium propionate retarded the growth of molds when applied to figs and berries. On shelled peas and lima beans, it maintained their good quality which was often lost as a result of the delay between harvesting and processing, during which bacterial activity was high.

Schroeder (1964) found that the rate of infection by storage fungi, especially Aspergillus candidus, on rough rice (Oryza sativa) was significantly reduced by treatment with sodium propionate.

Often, if parchment wrappers are impregnated with the preservative, mold and bacterial growth can be inhibited to an appreciable extent on food products. Propionates are also used to suppress the growth of molds in some chewing and smoking tobaccos.

In the past few years propionic acid, in combination with

acetic acid has been used successfully in preventing microbial deterioration of stored moist grain. In North America the mixture is called CHEMSTOR. In the United Kingdom the product which is based only on propionic acid is called PROPCORN.

Harvey (1967) gives several reasons for storing damp grain. Harvest can be conducted earlier and when wet. Also grain can be salvaged from damage by natural elements, particularly wind. Moreover, he suggests that grain should be moist when used as feed for livestock. Dry feed is dusty and after grinding contains a large proportion of unbroken grains. Since it is unpalatable in this form, it may cause digestive upset or is wasted as it passes through the animal. He states that investigations have been conducted demonstrating higher intake and higher live weight gains in animals fed moist barley.

Ingoing grain, that is grain for storage, shows a mixed microbial flora including bacteria, fungi, actinomycetes as well as the spores of bacteria and fungi. The types are obviously those existing in the soil, air and on dead or living plants or animals. They occur both inside and on the grain surface and thus are commonly referred to as internal or external microflora (Anderson and Alcock, 1954).

Hyphae of molds such as those of Alternaria, Aspergillus, Cladosporium, Fusarium, Helminthosporium, Mucor, Penicillium, and Rhizopus have been identified as the main internal grain microorganisms. Articles by Warnock (1971) and Warnock and Preece (1971) discuss recent methods of identifying, locating, and determining the extent of fungal mycelia in grains of barley. The above cited species also occur externally.

In addition, Candida, Monilia, Saccharomyces and Torula are well known yeast-like fungi usually found on grain surfaces. Bacteria include species of Acetobacter, Aerobacter, Bacillus, Clostridium, Escherichia, Flavobacterium, Lactobacillus, Micrococcus, Proteus, Pseudomonas and Serratia. Nichols and Leaver (1966) reported a bacterial population of 1-10 million per g of ingoing grain.

Damp grain is an excellent medium for the growth of the grain microflora. This can be hazardous to animals and humans as certain fungi, in particular Absidia, Aspergillus, Mucor and Penicillium release toxic substances which cause mycotic abortions, pulmonary aspergillosis and abdominal ulcers in livestock. Amongst handlers of such grain, "Farmer's Lung", due to the presence of a thermophilic Streptomyces, is not uncommon. Moreover, the action of these micro-organisms not only lowers the full feeding value but hampers the free flowing properties of the grain upon removal from storage.

To curb this problem, drying and refrigeration have been employed especially by the large cereal growers. There is, however, another alternative. Wet grain with 25-30% moisture content can be stored in sealed containers called "silos" in which air circulation is cut off. With the lack of oxygen, fermentation of plant material takes place producing an acid environment and formation of silage. In order for this method to be effective, the silo must be constructed so as to prevent any leakage of air during operation. This is very expensive since it involves a built-in system consisting of an unloading auger and a device to control changes in air pressure as the volume of the grain in the silo falls. Nevertheless, even under such controlled conditions, 15 to 40% losses in ensiled crop have been reported.

Several studies have been carried out to determine the types of microorganisms existing on damp grain, mainly barley as it is used for feed (Christensen, 1957; Mulinge and Chesters, 1970a and b; Nichols and Leaver, 1966). The types of predominant microorganisms depend on the growth requirements of these microorganisms in relationship to their environment. Often the storage fungi, Absidia, Aspergillus and Penicillium appear even if not initially discovered on ingoing grain. It has been postulated (Christensen, 1957) that their development could be related to insect infestation, but handling could also have introduced mold spores.

The preservative effect of propionic acid on moist grain is not an entirely novel idea. Richardson and Malick (1958) showed that propionic acid decreased heating and the growth of molds in yellow corn (17% moisture content) which was eventually to be used for feed.

Chemical preservation with propionic acid has many advantages over drying, refrigeration and silage-making (Huitson, 1968; Smedley, 1969). Treated grain can be stored up to one year without fear of further microbial growth. Since the acid is absorbed by the grain, the preservative action persists and the treated grain can be transported and stored again in bulk without deterioration. Moreover, it can be rolled, ground and stored, thereby eliminating daily grinding.

The acid is applied by spraying freshly harvested grain as it enters an auger where continuous mixing ensures a thorough distribution. This is essential as any pocket of untreated grain could heat up as a result of microbial growth. The rate of application is determined by the moisture content of the grain as well as the grain carrying capacity of the auger. The treatment rate has been shown to

be directly proportional to the grain moisture content. A maximum output of approximately 20 tons of treated corn per hour has been attained (Wray, 1969) exemplifying the efficiency of the system.

There are no specific storage restrictions on treated grain, except that it should not be in contact with water. Propionic acid is a weak organic acid and is therefore progressively corrosive to certain metals such as iron and zinc. Concrete and other cement products which are alkaline in nature are also attacked. To by-pass this, protective coatings can be used. The cheapest and most durable is polyethylene sheeting which can be placed on any surface and in addition, prevents the grain from becoming wet or damp.

Results of feeding trials on all types of livestock have shown that the preservative is safe. Meat and milk quantity and quality are not affected. Palatability and intake remained normal while growth and gain rate were equal or improved.

However, there are a few inconveniences. Propionic acid treated grain is destined only for animal feed. Seed grain and barley for malting must not be treated since germination is arrested. Also, the levels utilized are too high for human consumption.

II. The Mode of Action of Propionates

Ingram, Ottaway and Coppock (1956) cite three aspects that cannot be overlooked when dealing with acid preservatives: effect of pH, dissociation, and specific effects. The effect of hydrogen ion concentration on growth inhibition can be seen by adding equal amounts of weak and strong acid to two systems, unbuffered and buffered. Addition of strong acid to the unbuffered medium gives a low pH and growth is inhibited while with

the weak acid, there is a slight change in pH and growth is little affected. In the buffered system where the pH of the medium is constant, the weak acid is more inhibitory than the strong acid. This suggests that weak acids act chiefly in the undissociated form and is substantiated by the fact that when a substance to be treated is acidified, the amount of salt or acid required for growth inhibition is reduced. Undissociated acid molecules are uncharged and penetrate cells more readily than charged molecules.

In regards to specific effects, organic acids could behave like surface active agents and disrupt cell membranes. Alternatively, they may interfere with essential metabolic pathways.

Heseltine (1952) proposed that on the basis of structural similarity between propionic acid and the amino acid DL - α - alanine, propionates might act as substrate competitors of amino acids. However his data did not confirm this. He did suggest that bacteriostatic action was produced by the molecule rather than the ion, and that the site of action was on carbohydrate metabolism.

The first evidence that propionate interfered with aspects of the pyruvate dehydrogenase complex was supplied by Hill (1953). He showed that inhibition of Streptococcus faecalis growth by propionate prevented the formation of acetate from pyruvate and could be reversed either by the addition of acetate or pantothenate to the growth medium. Recently, Kamihara, Yabushita and Fukui (1968,1969) have submitted substantial evidence that sodium propionate is activated to propionyl coenzyme A, thereby averting the generation of acetyl coenzyme A from pyruvate and subsequent formation of lipids apparently essential for growth of Streptococcus faecalis. Results of experiments which lead

to this assumption were: propionic acid inhibited lipoate dependent aerobic oxidation of pyruvate by cell suspensions and cell free extracts. This inhibition was alleviated by the addition of more lipoic acid. Greater inhibition was achieved with the activated form, propionyl phosphate, since it was observed that propionate was converted to propionyl coenzyme A via the phosphate ester. Propionate inhibition of aerobic oxidation of pyruvate was reversed by addition of acetate which also caused a marked decrease in the amount of propionyl coenzyme A formed and the appearance of acetyl coenzyme A. Inhibition of growth by propionate was reversed by certain lipids and when acetate - 2 - ^{14}C was incorporated into cells, radioactivity was detected in the lipid fraction.

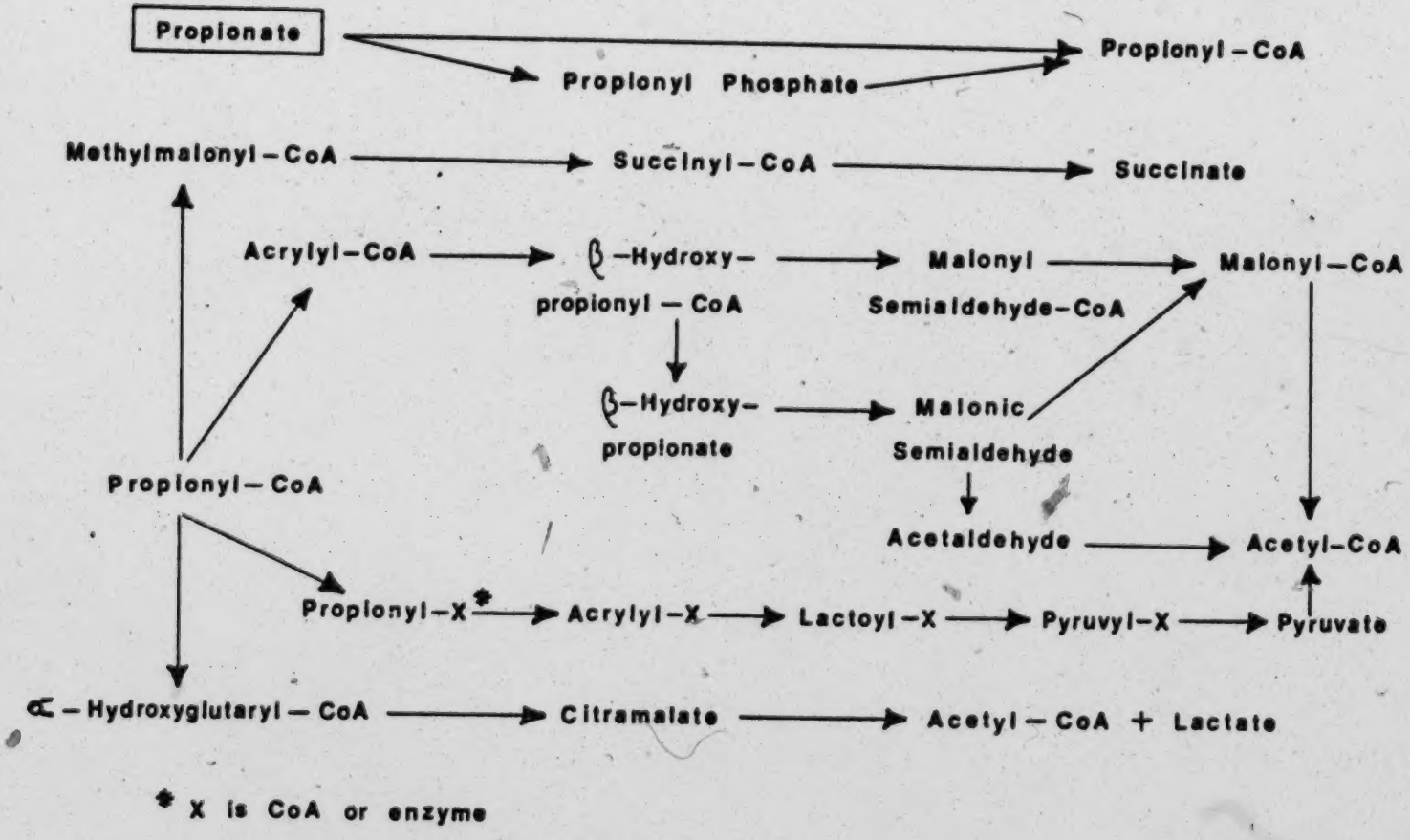
Since reversal of growth inhibition of a Saccharomyces species was obtained with only acetate and not lipoic acid, it was proposed that the mechanism of inhibition varied among different microorganisms, depending on the pathway of pyruvate oxidation (Kamihara, Yabushita and Fukui, 1968).

III. The Pathways of Propionate Metabolism

A summary of the known pathways of propionate metabolism is illustrated in Figure 1. The primary step involves the activation of propionate and conversion to propionyl coenzyme A from which diverge many routes. Succinate is the principal pathway by which propionate is oxidized in mammalian tissues (Kaziro and Ochoa, 1964). It involves subsequent carboxylation of propionyl coenzyme A to methylmalonyl coenzyme A which is then isomerized to succinyl coenzyme A. A few microorganisms utilize this pathway such as

FIGURE 1

KNOWN PATHWAYS OF PROPIONATE METABOLISM



* X is CoA or enzyme

Rhodospirillum rubrum (Knight, 1962), Micrococcus denitrificans (Smith and Kornberg, 1967), Ochromonas malhamensis (Arnstein and White, 1962), and Brevibacterium (Vestal and Perry, 1969).

The malonic semialdehyde pathway whereby propionyl coenzyme A is converted to malonic semialdehyde via acrylyl coenzyme A, β-hydroxypropionyl coenzyme A and free β-hydroxypropionate was proposed by Rendina and Coon (1957). It occurs mainly in plant tissues and is the major pathway of propionate metabolism in an alga, Prototheca zopfii (Callely and Lloyd, 1964).

The direct oxidation of propionate via acrylate to lactate or their acyl coenzyme A esters as intermediates, and thence to pyruvate, is commonly referred to as the acrylate or lactate pathway. It has been demonstrated in Clostridium propionicum (Leaver, Wood and Stjernholm, 1955) as well as in animal tissues (Mahler and Huennekens, 1953). It has been implied that enzyme bound intermediates are participating in Moraxella lwoffi (Hodgson and McGarry, 1968a and b).

In the malonyl semialdehyde coenzyme A pathway, β-hydroxypropionyl coenzyme A is oxidized to malonyl semialdehyde coenzyme A and thence to malonyl coenzyme A from which acetyl coenzyme A is formed. Clostridium kluyveri (Vagelos and Earl, 1959), appears to employ this pathway. In the α-hydroxyglutarate pathway, propionate, after activation, condenses with glyoxylate to yield α-hydroxyglutarate which is then cleaved to produce acetate and lactate, the latter being converted to pyruvate. It has been postulated (Wegener, Reeves and Ajl, 1968a and b) that this route is present in Escherichia coli E-26V which has adapted to growth on propionate. The glyoxylate is

probably derived from isocitrate, the cleavage being catalysed by isocitrate lyase.

Any of these pathways may be involved in the utilization of propionate by such a heterogenous population of microorganisms found on grain surfaces.

MATERIALS AND METHODS

I. Grain Samples

Grain samples were obtained from grain elevators in the vicinity of Edmonton. These elevators store grain which has been dried in the field to a moisture content of around 15%. Upon arrival at the elevators these grains are inspected and loads of similar classifications are pooled and stored.

For the purpose of obtaining reproducible results, it was decided to work with one specific barley sample. Until the supply was exhausted a sample of registered seed of the Paragon variety served in the majority of chemical tests and whole barley from Maple Leaf Mills was used in the radiorespirometric investigations.

II. Sources of Chemicals

All chemicals used were obtained from commercial sources, mainly the Fisher Scientific Company, and were of reagent grade. There were, however, a few exceptions as in the case of acetic acid and ammonium hydroxide which were purchased from the Allied Chemical Company of Canada, Limited.

Acetic acid - 1 - ^{14}C and sodium propionate - 1 - ^{14}C were obtained from the International Chemical and Nuclear Corporation.

Liquid scintillation fluids were prepared from their component chemicals (Bray, 1960; Woeller, 1961; Wang and Willis, 1965). Source of phenethylamine was the Eastman Company as well as Anachemia Chemicals Limited. PPO was purchased from Fraser Medical Supplies Limited and POPOP from Nuclear Enterprises Limited. Naphthalene was supplied by Amersham Searle.

III. Culture Media

Several media were evaluated for culturing grain microorganisms before selecting a standard plating medium for use throughout this study. It was found that PCA (Difco Laboratories), supported the growth of the greatest number and variety of colonies and was thus used in all routine plating work.

IV. Procedure for the Isolation and Enumeration of Grain Microflora

The method consisted of adding 10 g of grain aseptically to 90 ml of sterile distilled water. Dispersion of the surface grain microorganisms was accomplished by shaking vessels on an Eberbach reciprocal shaker for 20 min at 278 reciprocal movements per min, a procedure which was strictly adhered to in all subsequent investigations. For total viable count determinations which included bacteria, yeasts, molds, and actinomycetes, the spread plate method was used whereby 0.1 ml aliquots of serial dilutions were plated onto each of 5 PCA plates. These were then incubated for 24 hr at 30-33°C and afterwards, left at room temperature up to a period of 3 days or more, and counted using a New Brunswick colony counter.

V. Preparation of Test Solutions

There were essentially two types of test solutions, acid and salt. Due to the volatility of the fatty acids, the volume of water required to constitute 100 ml of an acid solution of a desired molarity was autoclaved first. Upon cooling, the calculated amount of stock acid was then added.

Salt solutions were prepared in 100 ml volumetric flasks and transferred to dilution bottles for autoclaving.

Ten ml aliquots from both acid and salt solutions were withdrawn for pH readings which were recorded by a Hach - Simpson Model 108, Number 28 pH meter with a Calomel electrode.

The effect that acid and salt solutions had on eliminating the microflora of 10 g of grain was determined after an incubation period of 24 hr at 30-33°C.

VI. Measurement of the pH of Aqueous Grain Extracts

Three replicates consisting of 2.5 g of grain for each grain sample were added aseptically to 18 x 150 mm sterile test tubes. Five ml of sterile distilled water were then added to saturate the grain. pH readings were taken after an equilibration time of 30 min.

VII. Assay of Radioactive Materials

Three fields of liquid scintillation counting were applied: aqueous solutions, samples dried on paper, and counting gases which involved trapping $^{14}CO_2$ in phenethylamine. Samples were contained in polyethylene counting vials and consisted of the radioactive material, an organic solvent or solvent mixture, and one or more organic phosphors. These were assayed by a Nuclear - Chicago Mark I, Model 6860 liquid scintillation counter. Optimal counter settings were:

Channel A: Alternator D: 7.9

Upper window: 9.9 (Maximum)

Lower window: 0.0 (Minimum)

Channel C: Alternator D: 7.9

Upper window: 2.2 (Maximum)

Lower window: 0.0 (Minimum)

Counting was carried out for 4 min and cpm were converted to dpm by the following equation:

$$dpm = \frac{cpm}{efficiency} \times 100\%$$

The efficiency was determined by the channels ratio method and averaged 85%.

VIII. Analysis of Radiochemical Purity

Purity of the two radioactive compounds was established by ascending paper chromatography of the ammonium salts of the fatty acids with a two phase solvent system comprising of n-butanol saturated with an equal volume of 1.5M ammonia. Chromatographs were developed by spraying with a 0.04% bromocresol purple solution of 1:5 v/v dilution of formalin in ethanol adjusted to pH 5 with 0.1N sodium hydroxide. Formaldehyde reacts with NH_4 forming a weak base, hexamethylenetetramine, thereby accentuating the acid spots (Block, Durrum and Zweig, 1958).

Spots were also located with a Nuclear Chicago Actigraph III, Model 1002, for labelled products. Actigraph settings were as follows:

Full scale: 1500 cpm

Speed: 30 cm/hr

Slit width: 6 mm

Flow rate: 6 psi

Both analyses confirmed the radiochemical purity of the two labelled acids.

IX. Steps Involved in the Preparation of $^{14}CO_2$ Production Studies

Articles by Wang and Willis (1965 and 1967) served as a guide. Figure 2 illustrates the construction of the $^{14}CO_2$ reaction vessel utilized in the course of the experiments.

Sterile grain was obtained by placing 25 g of untreated barley through a series of 100 ml washings consisting of: 95% ethanol (one wash), sterile distilled water (two washes), 1:1000 w/v mercuric chloride (one wash) and three of water in order to remove all traces of the mercuric chloride. The grain was then aseptically transferred to large sterile glass petri dishes lined with Whatman Number 1 filter discs, 15 cm in diameter and air dried in a laminar flow hood. To ensure sterility, several grains were aseptically placed onto PCA plates which were incubated for 24 hr at 30°C and then held at room temperature for 2 days. If any colonies were observed growing around the grain kernels, the entire 25 g batch was discarded. Seeds sterilized by this method seldom exhibited microbial growth and did not lose their ability to germinate.

Preliminary experiments indicated that the grain microflora inoculum should have a total initial viable count of approximately 10^9 cells /ml. To achieve this, a 15 g sample of untreated barley was added to 90 ml of 0.05% Bacto - Tryptone water and dispersed. This was repeated 5 times. The five grain supernatants were then pooled and incubated at 30°C for 24 hr while being agitated on a New Brunswick rotary shaker at 300 rpm (one inch eccentricity). After incubation, total viable count determinations were performed by the dilution plate method.

The radioactive concentration of stock sodium propionate

FIGURE 2

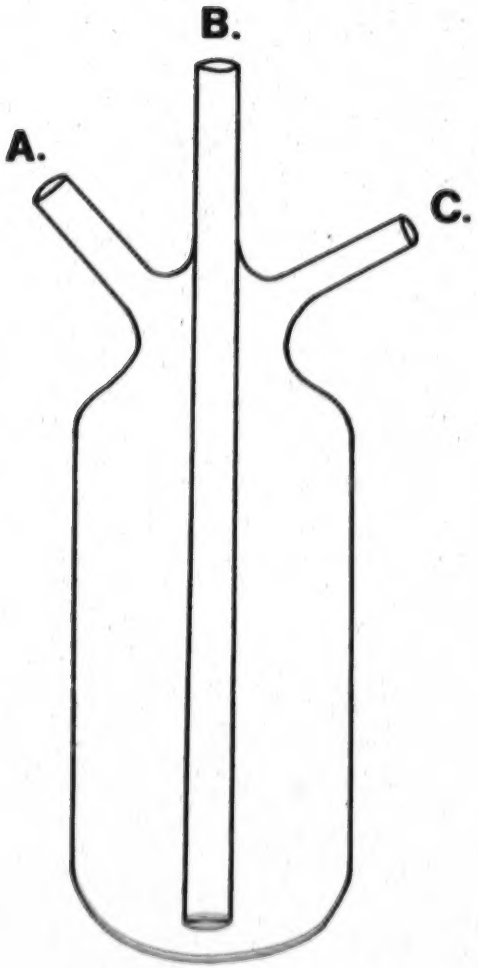
SCHEMATIC DIAGRAM OF $^{14}CO_2$ REACTION VESSEL

A. Grain and inoculum are admitted to the reaction vessel through this larger side arm which is then closed with a serum cap through which Bacto - Tryptone and labelled acid solutions are injected.

B. Sparging with air is accomplished through this glass tube fitted with rubber tubing leading to the air supply.

C. Any flushed $^{14}CO_2$ passes through this smaller side arm also fitted with rubber tubing, and through a Pasteur pipette immersed in 10 ml of phenethylamine scintillation fluid.

The reaction vessel conveniently accommodates 45 ml of reaction mixture, including an additional 5 g of grain when applicable.


B.
A.
C.

- 1 - ^{14}C was 1 mc/765 µl with a specific activity of 56.3 mc/mmol. On the other hand, the radioactive concentration of stock acetic acid - 1 - ^{14}C was 1 mc/2 ml with a specific activity of 10.5 mc/mmol. Appropriate quantitative transfers were carried out to obtain two working solutions for each of the labelled compounds with final radioactive concentrations of 1 µc/ml and 100 µc/ml. These working solutions were used to prepare 10 ml of 0.045M and 0.090M acetic and propionic acid solutions with radioactive concentrations of 1 µc/ml. Addition of 1 ml of these labelled acid solutions to 44 ml of reaction mixture would yield final concentrations of labelled acid solutions of 0.001M and 0.02M with a radioactive concentration of 1 µc/45 ml of reaction mixture.

Experiments were conducted at an incubation temperature of 30°C. The experimental set-up is shown in Figure 3.

X. Sampling Procedure

Due to its volatility, labelled acid was the last constituent added to complete the reaction mixture. Injection of acid was performed consecutively at 2 min intervals and sparging with air was carried out for 10 minutes. As soon as four, 1 ml plastic sterile syringes were filled with the appropriate labelled acid solutions and Pasteur pipettes were immersed in 10 ml of phenethylamine scintillation fluid, the radioactive solution was injected. The respective vessel was shaken manually for several seconds, the syringe was rinsed twice and 0.5 ml of the reaction mixture were withdrawn and placed in 5 ml of Bray's scintillation fluid. The count obtained depicted the initial amount of radioactivity

FIGURE 3

PHOTOGRAPH SHOWING EXPERIMENTAL SETUP FOR

$^{14}CO_2$ PRODUCTION STUDIES

A. Glass wool air filters.

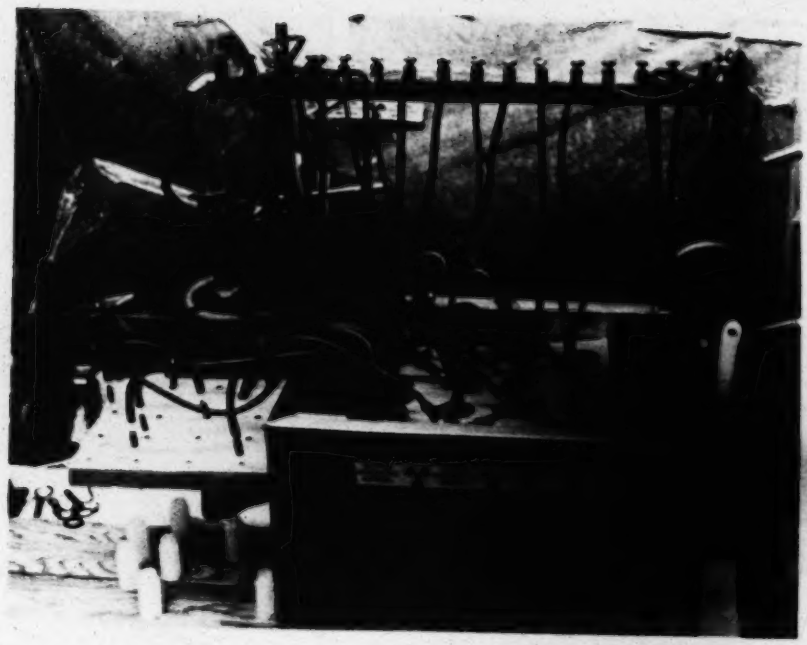
B. Air source.

C. Manifold conducting a regulated flow of air from a single source to reaction vessels.

D. $^{14}CO_2$ reaction vessels immersed in a 30°C water bath and supported by a styrofoam frame.

E. Pasteur pipettes transporting released $^{14}CO_2$ into 10 ml of phenethylamine scintillation fluid, also supported by a styrofoam frame which can be easily raised or lowered as required.

C.



E.

A.

B.

D.

added to the system and upon which all percentage calculations were based. After 2 min, the appropriate air cock was opened. Sparging with air was performed and repeated every hour thereafter for 6 hr, and then 24 hr after the initial injection of labelled acid.

Upon termination of the experiment, several steps were undertaken to account for total radioactivity as well as its distribution. To determine the amount of radioactivity remaining in the reaction mixtures, all reaction vessels were shaken manually and 10 ml transferred aseptically to sterile 18 x 150 mm test tubes. Each was mixed on a Vortex Genie for approximately 15 seconds and 0.5 ml was transferred with a sterile 1 ml pipette to 5 ml of Bray's solution.

Viable counts of reaction mixtures were then determined. From these reaction mixtures, 1.0 ml was also filtered through 0.45 micron millipore filters and washed twice with 1 ml of sterile growth medium. These filters were dried and counted in 10 ml of toluene scintillation fluid. The filtrate was also sampled and counted in Bray's solution. Thus the distribution of counts, that in solution and that in the inoculum was determined.

To obtain the proportion of radioactivity contained in the grain, reaction mixtures were filtered through Whatman Number 1 paper. The filtrates were then sampled and counted in Bray's solution and the grain was washed with 45 ml portions of wash media. After each wash, the filtrate was sampled and counted. The grain was ground in 30 ml of distilled water in a mortar and the mash quantitatively transferred with 25 ml of water to 250 ml centrifuge pots which were spun at

10,000 rpm for 10 min at +4°C in a Sorvall Superspeed RC2-B automatic centrifuge. The supernatants were decanted and counted in Bray's. The grain mash was dried and 1 g was counted in 10 ml of toluene scintillation fluid.

RESULTS AND DISCUSSION

I. Analysis of the Dilution-plate Method for the Isolation and Enumeration of Grain Microflora

Several aspects of the dilution-plate technique warranted investigation. Milk dilution bottles with their elongated rubber stoppers were satisfactory for the dispersion of microorganisms from the surface of grains and air pockets formed within the husks. What was to be examined was the manner in which these dilution blanks accommodating 10 g of grain and 90 ml of sterile distilled water had to be shaken so as to bring about maximum displacement of grain microorganisms. The two methods of shaking examined were fifty complete perpendicular manual movements and automatic reciprocal shaking at high speed, that is, 278 reciprocal movements per minute for a defined period of 20 min. After exposing one such grain suspension to the manual method and another to the automatic method as described above, dilutions were carried out for each shaken grain suspension and 0.1 ml aliquots of the dilution giving approximately 200 colonies per PCA plate were plated onto each of 10 PCA plates for the grain suspension subjected to the automatic method, and, 8 for the manually shaken one. Total microbial counts which included bacteria, yeasts and molds are presented in Table I. Data were analyzed by the Student two-tailed t-test for unpaired data (Appendix I). The calculated statistic ($t = 12.5$ for 16 degrees of freedom) was rejected at the 1% and 5% levels of significance indicating that there was a highly significant difference in total viable counts obtained from the two methods of dispersion. This was obvious upon direct observation of the numbers

TABLE I

COMPARISON OF METHODS OF DISPERSION

Viable Counts ($x10^{-7}$)/g of Grain	
Manual*	Automatic**
2.75	8.90
3.45	8.00
3.35	8.20
4.05	7.10
2.65	10.45
2.90	9.45
2.75	7.20
3.50	7.90
—	6.85
—	7.60

*Manual = 50 reciprocal manual shakings.

**Automatic = 278 reciprocations/min for 20 min. (The number of reciprocations per min for 20 min is accepted as optimal for mechanical shaking in the microbial ecology laboratory.)

but the statistical test was nevertheless carried out as an exercise in the application of statistics to microbiological data. Thus the automatic means of shaking was adopted as a standard and routine procedure throughout the study.

Although several media for total viable count determinations were tested, PCA was preferred for several reasons. Not only did colonies develop more rapidly on PCA, but the pigmentation of this medium offered a superior contrasting background for detecting late-developing white mycelia. As a recovery medium, PCA exhibited strong neutralizing activity. A surface pH of 5.5-6.0 was recorded when the number of survivors was being determined from a direct plating of 0.1 ml from the supernatant of grain treated with 2M acid.

The next step was to determine the reproducibility of total viable counts of 10 g samples taken from the same grain population. This was imperative as any significant discrepancy would indicate samples were not truely representative. Total viable counts on 5 PCA plates for each of four, shaken 10 g grain suspensions are illustrated in Table II. An F value of 5.20 (numerator degrees of freedom = 3, and denominator degrees of freedom = 16) was obtained when data were subjected to the F-test of variance for a completely randomized design. Viable counts were not significantly different at the 1% level. It was concluded that the microbial load was evenly distributed.

II. A Visual Test for Determining the Effectiveness of Grain Preservatives

A simple but very reliable test was developed for the livestock

TABLE II

VIABLE COUNTS OF SAMPLES FROM THE SAME GRAIN POPULATION

Sample	Viable Counts ($x10^{-7}$)/g of Grain				
I	6.05	6.75	5.65	5.60	6.50
II	6.65	8.90	7.60	9.45	10.45
III	3.80	4.45	5.90	8.00	6.60
IV	5.70	8.65	7.25	6.95	8.75

feeding industry to evaluate the effectiveness of treating harvested moist grain with acid to prevent the development of "hot spots", a manifestation of microbial activity. All this test involved was adding 10 g of the treated grain to 90 ml of sterile 0.05% Bacto - Tryptone water, shaking the mixture and incubating it at room temperature for a minimum period of 48 hr. Grain kernels were seen settling to the bottom of the vessel. The clarity of the supernatant determined whether the acid was indeed successful in killing grain microorganisms.

Similarly, this test was applied to establish what concentration of the preservative acid was required to sterilize the grain. Once again, 10 g of grain, but untreated in this case, were added to 90 ml of the desired concentration of acid containing 0.05% w/v of Bacto - Tryptone. Vessels were then shaken automatically for 20 min and incubated at 30°C for 24 hr. Effective concentrations of the preservative prohibited visible growth yielding clear supernatants. After shaking the vessels again for 20 min, total viable count determinations were carried out by adding 0.1 ml of the supernatant onto each of five PCA plates which were incubated for 24 hr at 30°C and then left at room temperature for at least 3 days. Biocidal concentrations of acid did not permit the growth of any bacteria, mold or yeast colonies on this recovery medium whereas biostatic concentrations did. Typical results are presented in Figure 4.

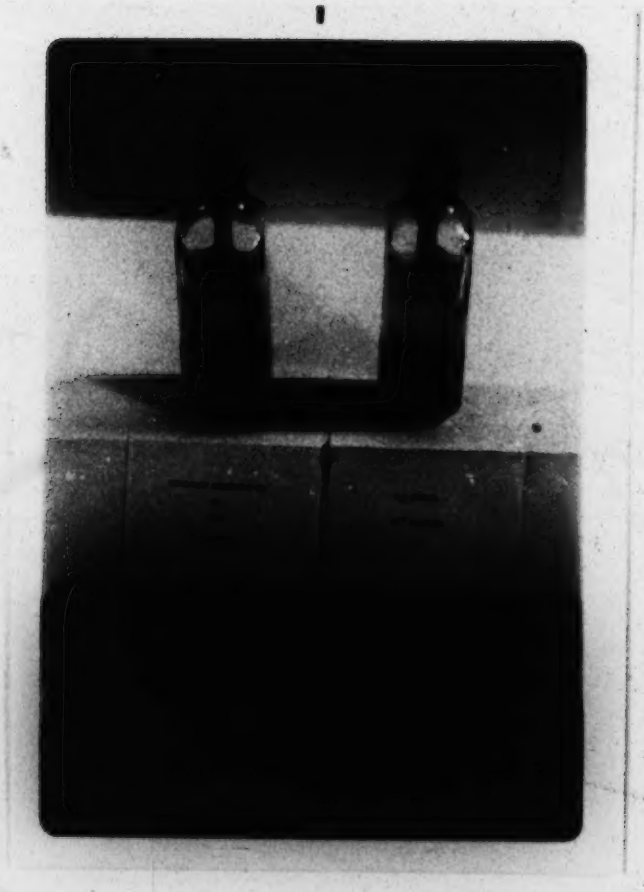
The importance of this elementary test cannot be overemphasized and it is recommended for use by personnel in the livestock feeding industry.

FIGURE 4

A SIMPLE TEST FOR DETECTING GRAIN

STERILIZING CONCENTRATIONS OF PRESERVATIVE ACID

The bottles contained 10 g of untreated grain each and 90 ml of 2M propionic acid (left) or 90 ml of water (right). After incubation for 24 hr at 30°C the supernatant in the bottle containing .05% Bacto - Tryptone water and grain was turbid, whereas the supernatant in the bottle containing propionic acid and grain was clear. The supernatants were analysed for viable organisms by plating 0.1 ml of the appropriate dilutions.



III. Effect of Acid Concentration on Microbial Death

The grain preservative is supplied as a mixture of propionic and acetic acids. These mixed acids are a by-product of an industrial chemical process. Standard dose-response curves were obtained for each acid for the purpose of examining their individual roles in preservation. In addition, a similar curve for hydrochloric acid was obtained to compare the effects of the weak acids to those of a strong one.

The number of survivors at concentrations of acid ranging from $5x10^{-5}$ M to 2M were determined. To obtain a linear relationship between these two variables, data were transformed to $\log_{10}$. Results are shown in Figure 5. A linear relationship was not evident at concentrations higher than $1x10^{-2}$M. Thus the best fitting geometric curve was plotted according to the least square method (Appendix I) only up to this value. All three acids demonstrated similar effects at identical concentrations. A control consisting of 10 g of untreated grain and 90 ml of water gave a viable count of $1.1x10^{8}$/g. Since the initial viable count was $8.2x10^{7}$/g, minimal growth had occurred. No colonies were observed when grain was treated with 2M acetic and propionic acids.

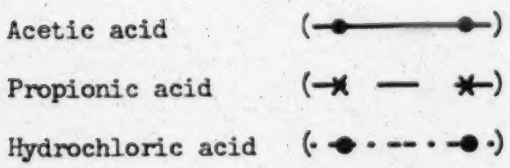
This introduced the problem of determining which property of a weak organic acid was responsible for the killing of grain micro-organisms: the concentration of hydrogen ions, dissociated, or undissociated acid.

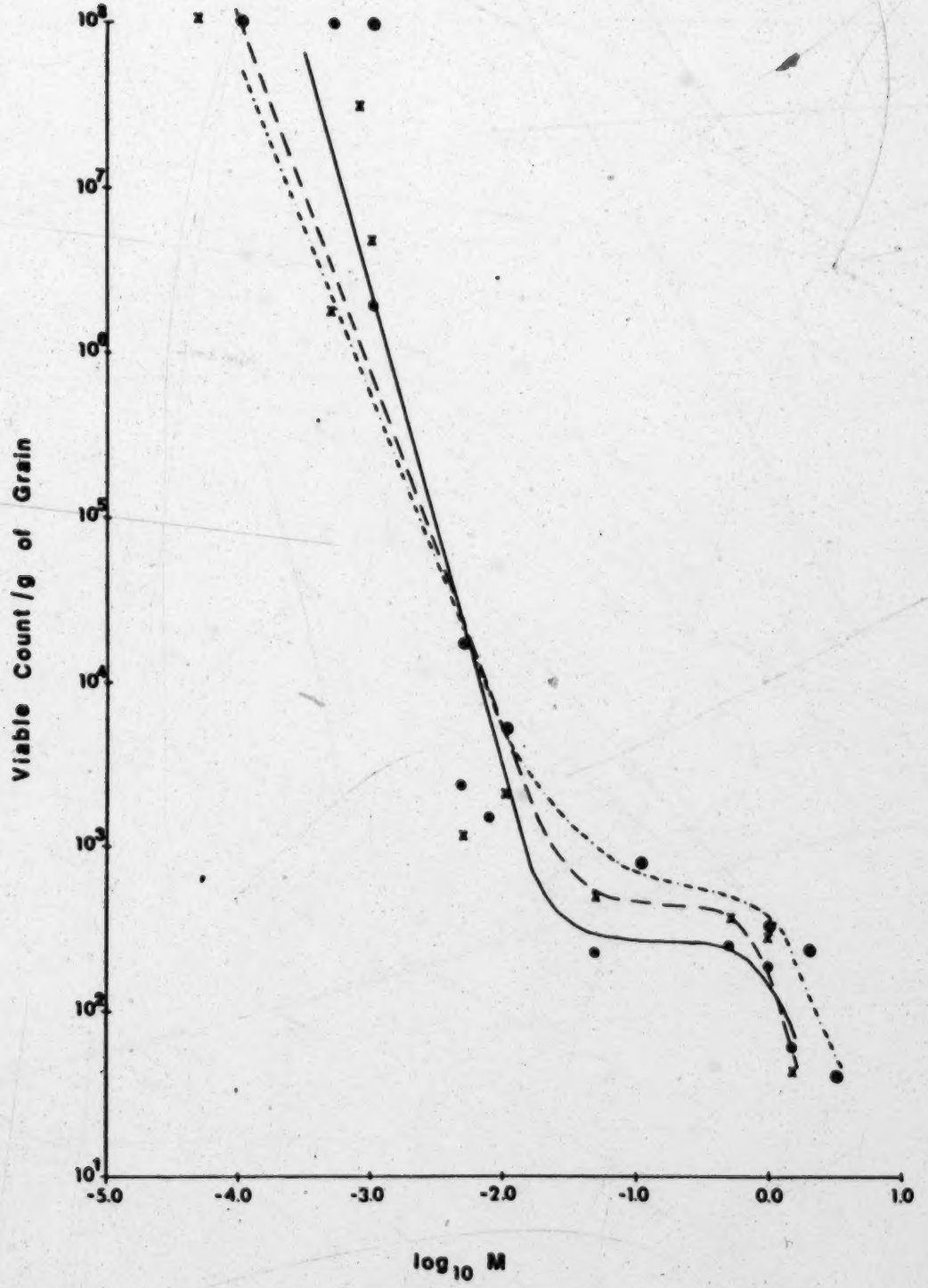
When comparing the effects of these three acids on the basis of

FIGURE 5

VIABLE COUNTS OF GRAIN AT INCREASING CONCENTRATIONS OF ACETIC, PROPIONIC AND HYDROCHLORIC ACIDS

Ten g of untreated grain (viable count/g = 8.2×10^7) were mixed with 90 ml of acid of various concentrations. After incubation at 30°C for 24 hr, mixtures were shaken and 0.1 ml samples of the appropriate dilutions analysed for viable organisms.




Viable Count /g of Grain
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
-5.0
-4.0
-3.0
-2.0
-1.0
0.0
1.0
$\log_{10}$ M

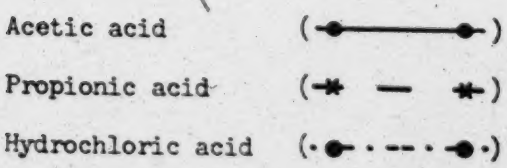
pH alone, Figure 6, the organic acids were significantly more effective than hydrochloric acid at identical hydrogen ion concentrations: exponential curves were fitted according to the least square method (Appendix I). The control exhibited a pH of 6.87. Acetic acid was biocidal at pH 2.26 and propionic acid at pH 2.40. Thus the killing effect exhibited by these two organic acids could not be entirely a function of pH but also must be attributed to the nature of the molecule which, in turn, exists in the dissociated and undissociated state. These observations are consistent with those of Ingram, Ottaway and Coppock (1956). The insignificant difference observed in the killing effects by these three acids at similar concentrations, Figure 5, can be explained by the fact that since hydrochloric acid is a strong acid and completely dissociated, at identical concentrations it will exhibit lower pH values than either of the two weakly dissociated organic acids. This is illustrated in Figure 7.

If pH was entirely responsible for the killing effect, then a buffer system of known pH would be expected to perform similarly to solutions of acid alone at similar pH. Data presented in Tables III and IV disprove this hypothesis. At all pH values, the acetate and propionate buffers were significantly more effective than their respective acid solutions of similar hydrogen ion concentration. Examination of the concentration of undissociated acid shows that at similar pH values, the concentration of undissociated acid for the buffers is much greater than that of the acid solutions. On the other hand, that this greater killing ability exhibited by the buffers is due to the constituting amount of salt cannot be overlooked. Figures 8 and 9 show the individual killing effects of acid and salt comprising 2M

FIGURE 6

INFLUENCE OF pH ON TOTAL VIABLE COUNTS FOR ACETIC, PROPIONIC AND HYDROCHLORIC ACIDS

Ten g of untreated grain (viable count = 8.2×10^7/g) were mixed with 90 ml of acid of known pH. After incubation for 24 hr at 30°C, 0.1 ml samples of the appropriate dilutions were analysed for viable organisms.



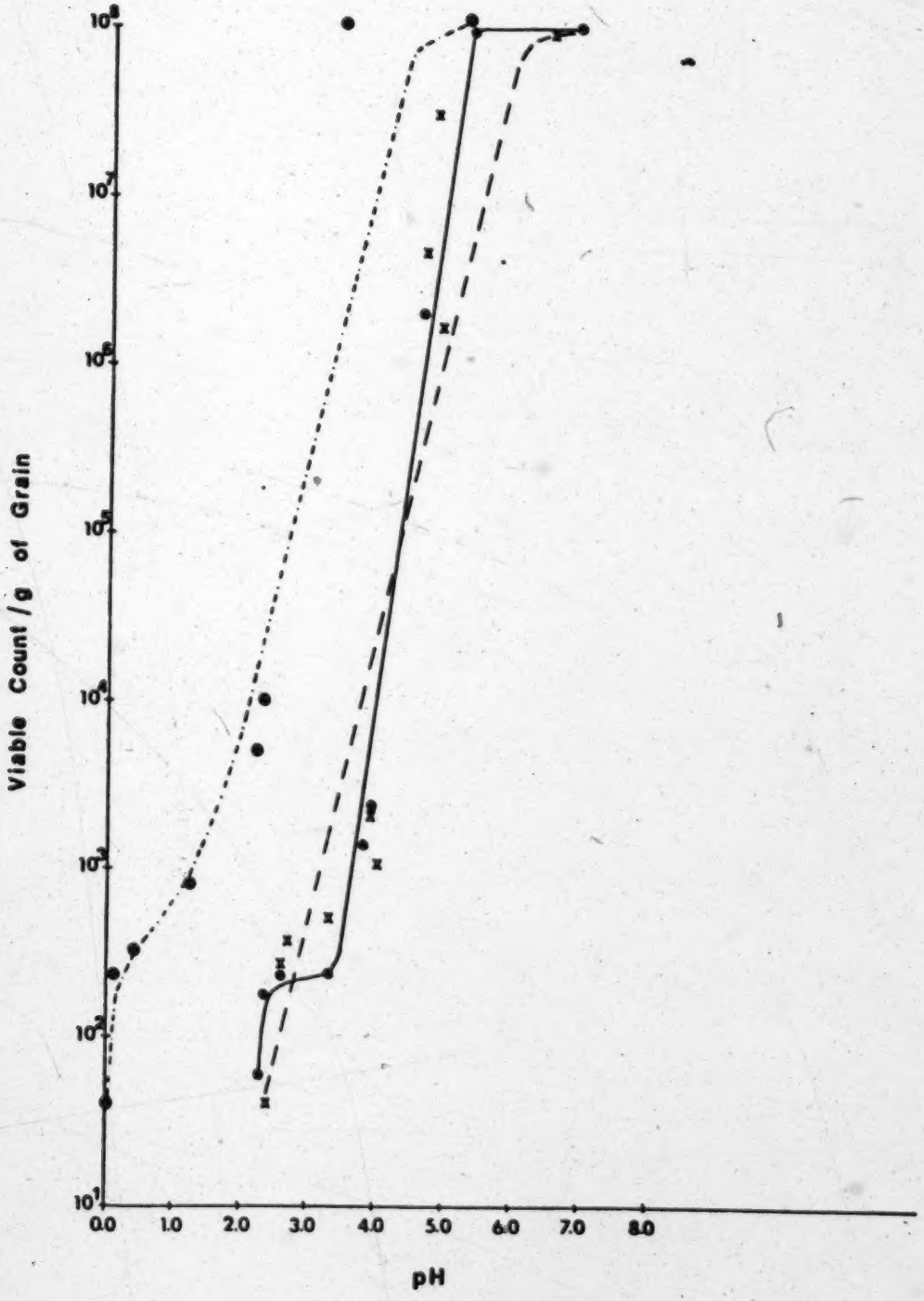
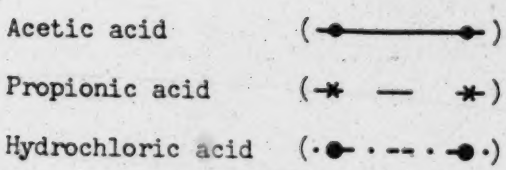

Viable Count /g of Grain
pH
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0

FIGURE 7

A COMPARISON OF pH VALUES

AT EQUIVALENT CONCENTRATIONS OF

ACETIC, PROPIONIC AND HYDROCHLORIC ACIDS



Molarity
10^{1}
10^{0}
10^{-1}
10^{-2}
10^{-3}
0
1
2
3
4
5
pH

TABLE III

COMPARISON OF PRESERVATIVE EFFECTS OF ACETATE BUFFERS TO THAT OF ACETIC ACID AT SIMILAR pH VALUES

	[Undissociated Acid] (M)		Viable Count/g of Grain	
pH	Acetic acid	*Acetate buffers	Acetic acid	*Acetate buffers
2.86	1.12×10^{-1}	1.97	2.3×10^{2}	3.3×10^{1}
3.83	1.28×10^{-3}	1.73	3.5×10^{3}	3.3×10^{1}
4.30	1.43×10^{-4}	1.61	8.0×10^{4}	6.0×10^{1}
4.50	6.22×10^{-5}	1.30	3.5×10^{5}	6.0×10^{1}
4.80	1.46×10^{-5}	9.6×10^{-1}	3.0×10^{6}	8.0×10^{1}
4.92	8.20×10^{-6}	7.4×10^{-1}	6.0×10^{6}	3.0×10^{2}
6.02	5.10×10^{-8}	1.1×10^{-1}	1.0×10^{8}	7.7×10^{2}
6.97	$< 10^{-9}$	1.0×10^{-2}	1.0×10^{8}	7.0×10^{2}

The concentration of undissociated acid was calculated from the Ka expression and the Henderson - Hasselbalch equation (Appendix II).

*The concentration of the acetate buffers at the specified pH values is 2M.

TABLE IV

COMPARISON OF PRESERVATIVE EFFECTS OF PROPIONATE BUFFERS TO THAT OF PROPIONIC ACID AT SIMILAR pH VALUES

	[Undissociated Acid] (M)		Viable Count/g of Grain	
pH	Propionic acid	*Propionate buffers	Propionic acid	*Propionate buffers
3.06	5.81×10^{-2}	1.97	6.0×10^{1}	**Ng
3.97	8.81×10^{-4}	1.77	2.5×10^{4}	1.0×10^{1}
4.30	1.92×10^{-4}	1.69	9.0×10^{4}	**Ng
4.56	5.82×10^{-5}	1.42	2.6×10^{5}	2.0×10^{1}
4.88	1.34×10^{-5}	1.10	1.3×10^{6}	1.0×10^{2}
5.05	6.13×10^{-6}	8.7×10^{-1}	1.7×10^{6}	3.3×10^{2}
5.82	1.75×10^{-7}	1.4×10^{-1}	3.7×10^{7}	6.0×10^{2}
7.15	$< 10^{-9}$	2.0×10^{-2}	1.0×10^{8}	9.7×10^{2}

The concentration of undissociated acid was calculated from the Ka expression and the Henderson - Hasselbalch equation (Appendix II).

*The concentration of the propionate buffers at the specified pH values is 2M.

**No growth, namely, no survivors.

FIGURE 8

ADDITIVE EFFECT OF SODIUM ACETATE AND ACETIC ACID ON THE GRAIN MICROFLORA

Ten g of grain (viable count = 8.2×10^7/g) were treated with 90 ml of 2M acetate buffers whose proportions of acid and salt were defined. Then 90 ml of these known concentrations of acid and salt comprising the 2M buffers were individually tested for their effects on 10 g of grain. After incubation at 30°C for 24 hr, mixtures were shaken and supernatants analysed for viable organisms by plating 0.1 ml of the appropriate dilutions.

Sodium acetate (-✕--------✕-)

Acetic acid (-●----------●-)

Acetate buffers (-+-·····-+-)

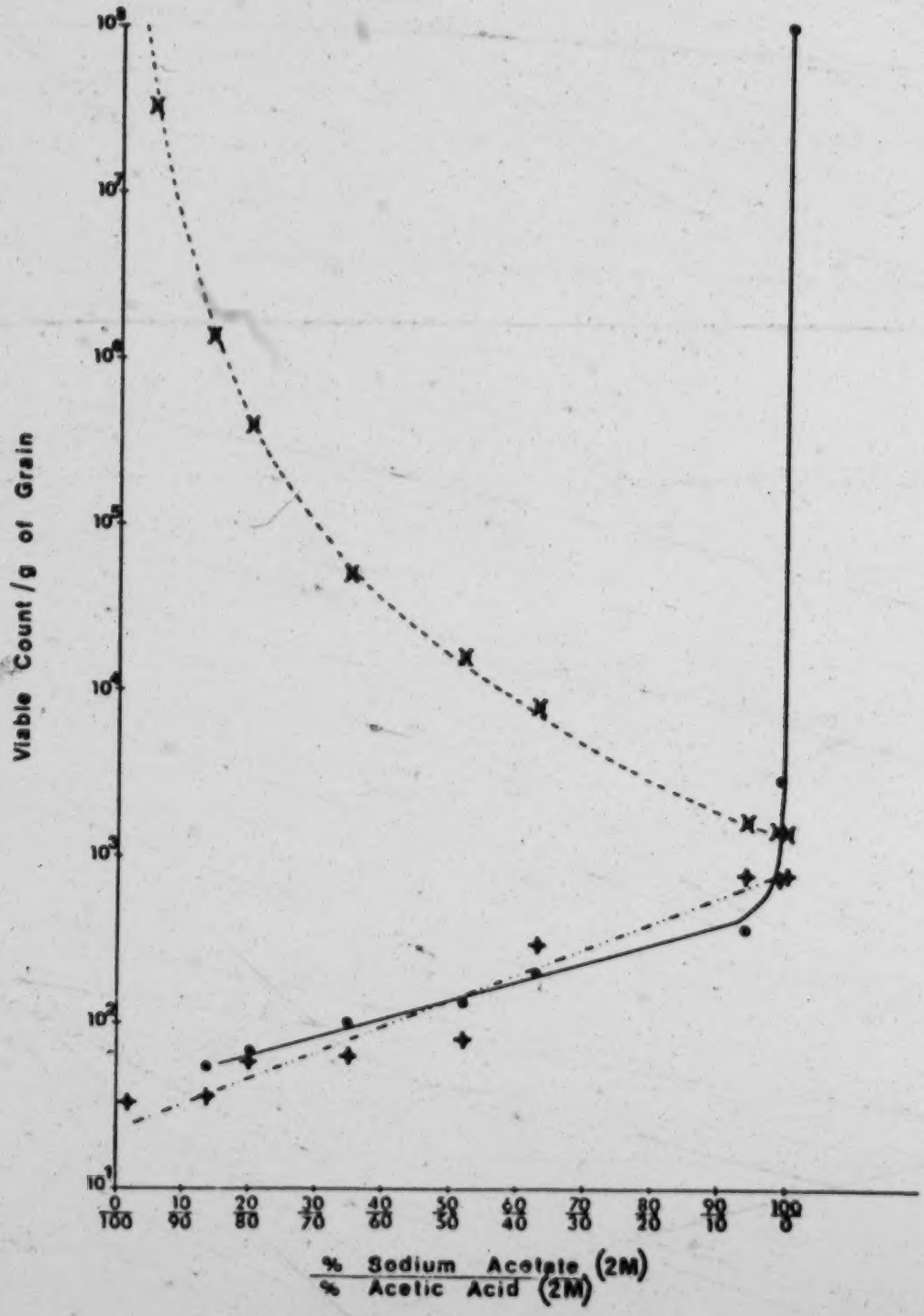

Viable Count / g of Grain
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
0/100
10/90
20/80
30/70
40/60
50/50
60/40
70/30
80/20
90/10
100/0
% Sodium Acetate (2M)
% Acetic Acid (2M)

FIGURE 9

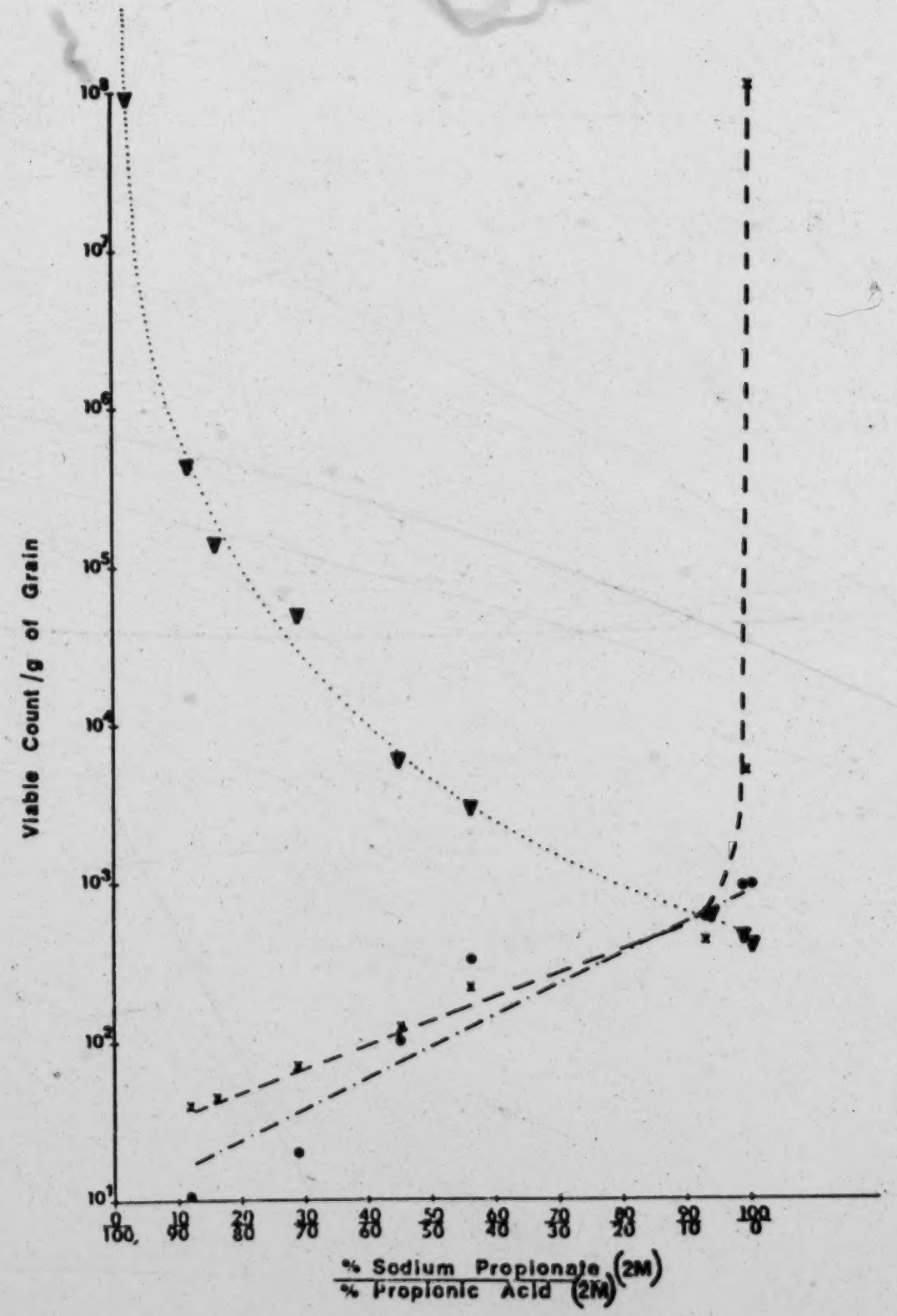
ADDITIVE EFFECT OF SODIUM PROPIONATE AND PROPIONIC ACID ON THE GRAIN MICROFLORA

Ten g of grain (viable count = 8.2×10^7/g) were treated with 90 ml of 2M propionate buffers whose proportions of acid and salt were defined. Then 90 ml of these known concentrations of acid and salt comprising the 2M buffers were individually tested for their effects on 10 g of grain. After incubation at 30°C for 24 hr, mixtures were shaken and supernatants analysed for viable organisms by plating 0.1 ml of the appropriate dilutions.

Sodium propionate (·▼·····▼·)

Propionic acid (✱ — ✱)

Propionate buffers (●·—·—·●)


Viable Count /g of Grain
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0 10 20 30 40 50 60 70 80 90 100
100 90 80 70 60 50 40 30 20 10 0
% Sodium Propionate (2M)
% Propionic Acid (2M)

acetate and propionate buffers of increasing pH in comparison to their additive sterilizing effects, namely, that of the buffers themselves. The additive sterilizing effects closely followed the killing effects of the comprising concentrations of acid which according to the Henderson-Hasselbalch equation (Appendix II) is the concentration of undissociated acid.

A more direct way of demonstrating that undissociated acid was responsible for killing grain microorganisms was to prepare a series of buffers of high pH such that their concentrations of undissociated acid matched those of their respective strong acid solutions of low pH readings. Results of such an experiment carried out at pH 4 are presented in Tables V and VI. These buffers gave the same number of survivors as the acid solutions at identical concentrations of undissociated acid.

Another approach showing the significance of the concentration of undissociated acid in preservation involved comparing the sterilizing effects of organic acids of corresponding molecular formulas and dissociation constants. Concentrated acid solutions were used since microorganisms may utilize these organic acids when present in small amounts. Acids with similar dissociation constants and molecular formulas were expected to exhibit the same number of survivors at identical concentrations. Table VII illustrates the results obtained. Viable counts were transformed to $\log_{10}$ and an analysis of variance performed on a completely randomized split-plot design which indicated a highly statistically significant difference between factors. Application of Duncan's new multiple-range test showed that viable counts of survivors obtained from the addition of

TABLE V

COMPARISON OF VIABLE COUNTS OBTAINED UPON INCREASING THE CONCENTRATION OF UNIONIZED ACETIC ACID FROM LOWER pH VALUES TO A pH OF 4

[Undissociated acid] (M)	Acetic Acid + Sodium Acetate			Acetic Acid	
	Total "acetate" (M)	pH	Viable count/g of grain	pH	Viable count/g of grain
2.14	2.5	3.98	*Ng	2.30	*Ng
1.73	2.0	3.83	3.3×10^{1}	2.35	6.0×10^{1}
1.28	1.5	3.80	1.6×10^{2}	2.40	1.5×10^{2}

*No growth, namely, no survivors.

TABLE VI

COMPARISON OF VIABLE COUNTS OBTAINED UPON INCREASING THE CONCENTRATION OF UNIONIZED PROPIONIC ACID FROM LOWER pH VALUES TO A pH OF 4

Propionic Acid + Sodium Propionate				Propionic Acid	
[Undissociated acid] (M)	Total "propionate" (M)	pH	Viable count/g of grain	pH	Viable count/g of grain
2.21	2.5	3.98	*Ng	2.20	*Ng
1.77	2.0	3.97	*Ng	2.33	*Ng
1.33	1.5	3.92	8.0×10^{1}	2.40	4.0×10^{1}
4.42×10^{-1}	5.0×10^{-1}	3.92	4.0×10^{2}	2.70	3.2×10^{2}

*No growth, namely, no survivors.

TABLE VII

STERILIZING EFFECTS OF ACIDS OF SIMILAR DISSOCIATION CONSTANTS

Acid	Molecular formula	Ka	pH of Solutions(M)			Average Viable Count/g of Grain at Acid Concentrations(M)		
			1	$1x10^{-1}$	$1x10^{-2}$	1	$1x10^{-1}$	$1x10^{-2}$
Acetic	CH_3COOH	$1.75x10^{-5}$	2.45	3.03	3.76	$4.4x10^{2}$	$1.0x10^{3}$	$1.0x10^{4}$
n-Butyric	$CH_3(CH_2)_2COOH$	$1.48x10^{-5}$	2.43	3.00	3.70	$2.0x10^{2}$	$1.4x10^{3}$	$3.2x10^{5}$
Propionic	CH_3CH_2COOH	$1.30x10^{-5}$	2.40	3.17	3.87	$2.6x10^{2}$	$1.4x10^{3}$	$3.4x10^{4}$
Formic	$HCOOH$	$1.76x10^{-4}$	1.89	2.40	2.95	$4.8x10^{2}$	$2.4x10^{3}$	$1.0x10^{3}$
Lactic	$CH_3CHOHCOOH$	$1.40x10^{-4}$	1.82	2.39	3.12	$2.4x10^{3}$	$9.8x10^{3}$	$2.9x10^{5}$

acetic, n-butyric and propionic acids to untreated grain at the designated molarities were not significantly different from each other at a 1% level of significance. A discussion of the usefulness of the statistics mentioned above is given in Appendix I. Although formic and lactic acids possess similar dissociation constants, and therefore the same concentration of undissociated acid at equivalent molarities, statistical analysis suggested that they exhibited significantly different killing effects on the grain microflora and that formic acid should be included with the other three acids. Perhaps the fact that formic acid is a smaller molecule, which could facilitate its cellular penetration as compared to lactic acid, could explain this discrepancy.

IV. Sterilization of Grain Resulting from Combining Acetic and Propionic Acids

The number of survivors was determined upon combining acetic and propionic acids in a 1:1 proportion to give final concentrations of $1x10^{-3}$M, $1x10^{-1}$M and 1M. Specifically, to obtain 100 ml of 1M combined acid, 3 ml of stock acetic acid (16.62M) and 3.8 ml of stock propionic acid (13.34M) were added to 93.2 ml sterile water. Ten ml were removed for pH readings. To the remaining 90 ml, 10 g of grain were added and the vessels accommodating these constituents were shaken. After an incubation period of 24 hr at 30°C, the number of survivors were determined by the dilution-plate method. Results obtained are presented in Table VIII. Data were transformed to $\log_{10}$ and an analysis of variance carried out for a completely randomized design for equal and unequal replication (Appendix I). The 5% and 1% levels of significance were used. There was no difference in the number of survivors when these acids were combined as compared to their individual killing effects.

TABLE VIII

RESULTS OF COMBINING ACETIC AND PROPIONIC ACIDS

IN RELATION TO INDIVIDUAL KILLING EFFECTS

Acid	Mean Number of Survivors at Molarities $1x10^{-3}$	$1x10^{-1}$	1.0
Acetic	$3.4x10^{7}$	$6.2x10^{3}$	$4.4x10^{2}$
Propionic	$1.4x10^{8}$	$3.5x10^{3}$	$4.6x10^{2}$
Acetic + Propionic	$3.2x10^{8}$	$4.2x10^{3}$	$1.3x10^{3}$

As mentioned previously, the grain preservative is supplied as a mixture of acetic and propionic acids because these mixed acids are a by-product of an industrial chemical process. Their separation could become an expensive and difficult undertaking and therefore impractical for the company, especially if these by-products are to be dispensed of in an inexpensive way. The results here indicate that their separation is not necessary.

V. Ability of Salts of Organic Acids to be Used as Preservatives

Figure 10 illustrates the killing effects of sodium acetate and sodium propionate at concentrations ranging from 5.2×10^{-2}M to 3.6M. The salts are not initially as effective as the organic acids (Figure 5) : at concentrations of 3.0×10^{-2}M of acid, viable counts were around 10^2/g of grain while at these concentrations of salt, viable counts were around 10^8/g of grain. Only at molarities greater than 1.0 do counts approximate each other. Data were converted to $\log_{10}$ to obtain a linear relationship between the variables.

The food industry prefers to use salts of organic acids in the preservation of food stuffs rather than the acids themselves for the simple reason that organic acids, and in particular acetic and propionic, are corrosive and very volatile and must therefore be handled with caution. As the results indicated, the sodium salts of acetic and propionic acids were not as effective in the sterilization of grain as the free acids.

VI. Studies Involving Time Curves

Time of exposure to a specific concentration of disinfectant is of considerable significance and studies with lethal agents have

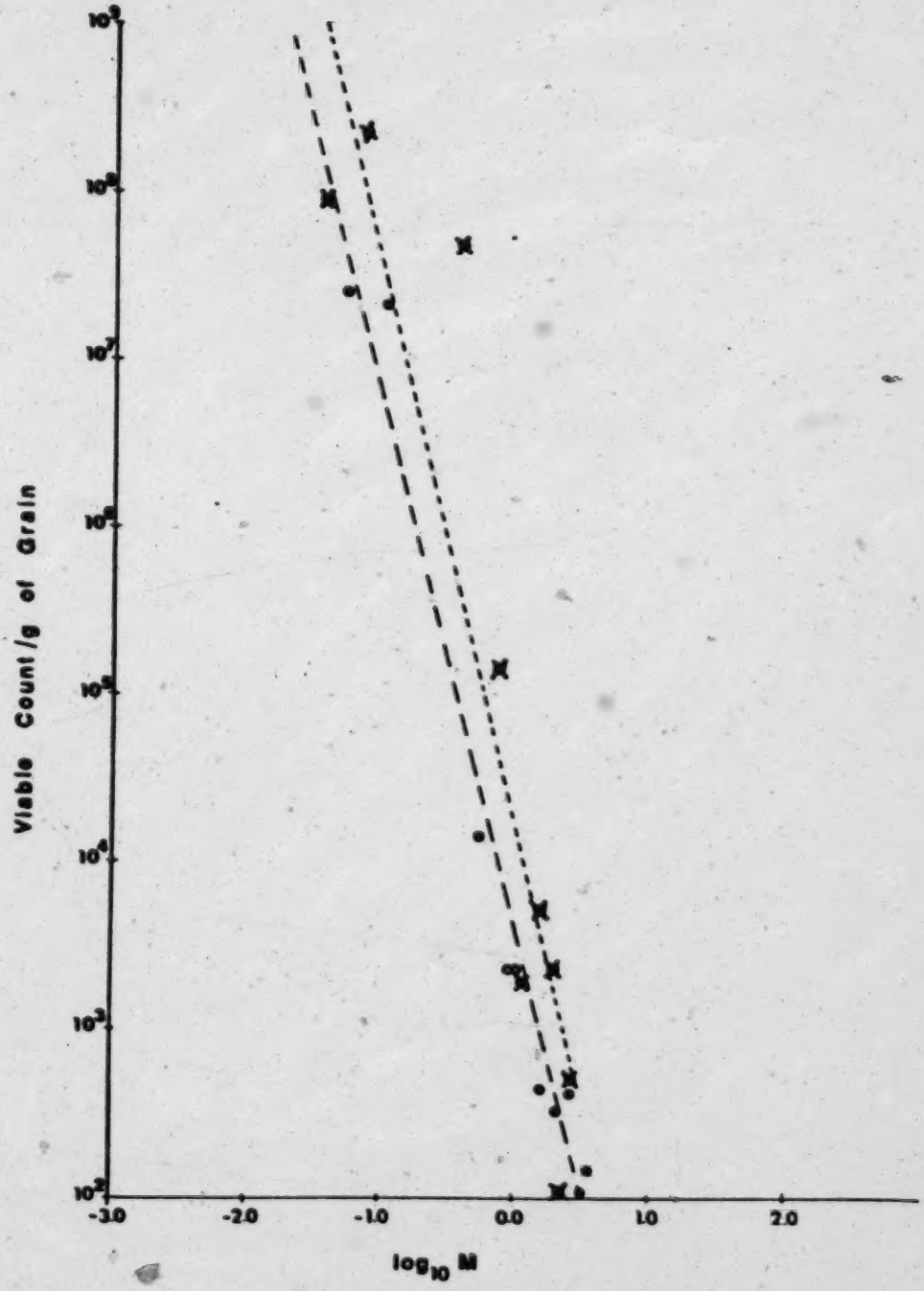
FIGURE 10

STANDARD DOSE-RESPONSE CURVES FOR SODIUM ACETATE AND SODIUM PROPIONATE

Ten g of untreated grain (viable count = 8.2×10^7) were added to 90 ml of salt solutions of known concentrations. After incubation at 30°C for 24 hr, 0.1 ml samples of the appropriate dilutions were analysed for viable organisms.

Sodium acetate (-✕-------✕-)

Sodium propionate (-o —— o-)


Viable Count/g of Grain
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
-3.0
-2.0
-1.0
0.0
1.0
2.0
$\log_{10}$ M

indicated that microorganisms die at a logarithmic rate.

A death rate curve was attempted with a concentration of propionic acid that was biocidal after 24 hr, namely 2M, in order to determine how quickly this concentration of preservative acid exhibited its killing effect on the grain microorganisms. Prior to the addition of stock acid required to constitute 90 ml of 2M acid, 10 g of untreated grain was added to the dilution bottle accommodating the desired amount of water. The vessel was shaken and placed in a 30°C water bath. Timing commenced as soon as stock acid was added and 0.1 ml aliquots of the grain supernatant were withdrawn aseptically and plated directly onto PCA at specified times up to a period of 1 hr. Average total viable counts of survivors for three replicates are presented in Table IX. The majority of killing took place within one minute of exposure to acid. No attempt was undertaken to obtain a death curve within the first minute of exposure. No survivors were observed after 24 hr of exposure to 2M propionic acid.

Figures 11 and 12 show the outcome of lengthier exposures to concentrations of acetic and propionic acids which demonstrated significant killing abilities after an exposure period of 24 hr, namely, $5x10^{-2}$M, $5x10^{-1}$M, 1M and 2M. At the designated sampling times, total viable count determinations of the shaken grain mixture were made by plating 0.1 ml aliquots of the supernatant or dilutions thereof, onto PCA plates. The biocidal effect of 2M acetic and propionic acids on the grain microflora after 24 hr of exposure was still evident after 44 days. The trace of growth observed with both acids at 2M on the sixth day of sampling is probably insignificant.

Being able to use a low concentration of acid which is biocidal

TABLE IX

KILLING OF GRAIN MICROFLORA DURING A ONE HOUR EXPOSURE TO 2M PROPIONIC ACID

Minutes of Exposure	Viable Count/g Grain
1	$2x10^2$
2	*Ng
3	$5x10^1$
4	$5x10^1$
5	$1x10^2$
10	$5x10^1$
15	$5x10^1$
20	$1.5x10^2$
25	$2.5x10^2$
30	$3x10^2$
35	$2x10^2$
40	$5x10^2$
45	$1x10^2$
50	$2x10^2$
55	$1.5x10^2$
60	$2.5x10^2$

Initial viable count/g of untreated grain was $8.2x10^7$.

*No growth, namely, no survivors.

FIGURE 11

GROWTH RESPONSE TO HIGHER CONCENTRATIONS OF ACETIC ACID AT INCREASING PERIODS OF INCUBATION

Ten g of untreated grain (viable count = 8.2×10^7) were added to 90 ml of the appropriate concentration of acid and after mixing, incubated at 30°C. At the indicated times, 0.1 ml samples of the supernatants were analysed for viable organisms.

2M concentration (—●————●—)

1M concentration (—● — ●—)

5×10^{-1}M concentration (—●--------●—)

5×10^{-2}M concentration (·●·····●·)

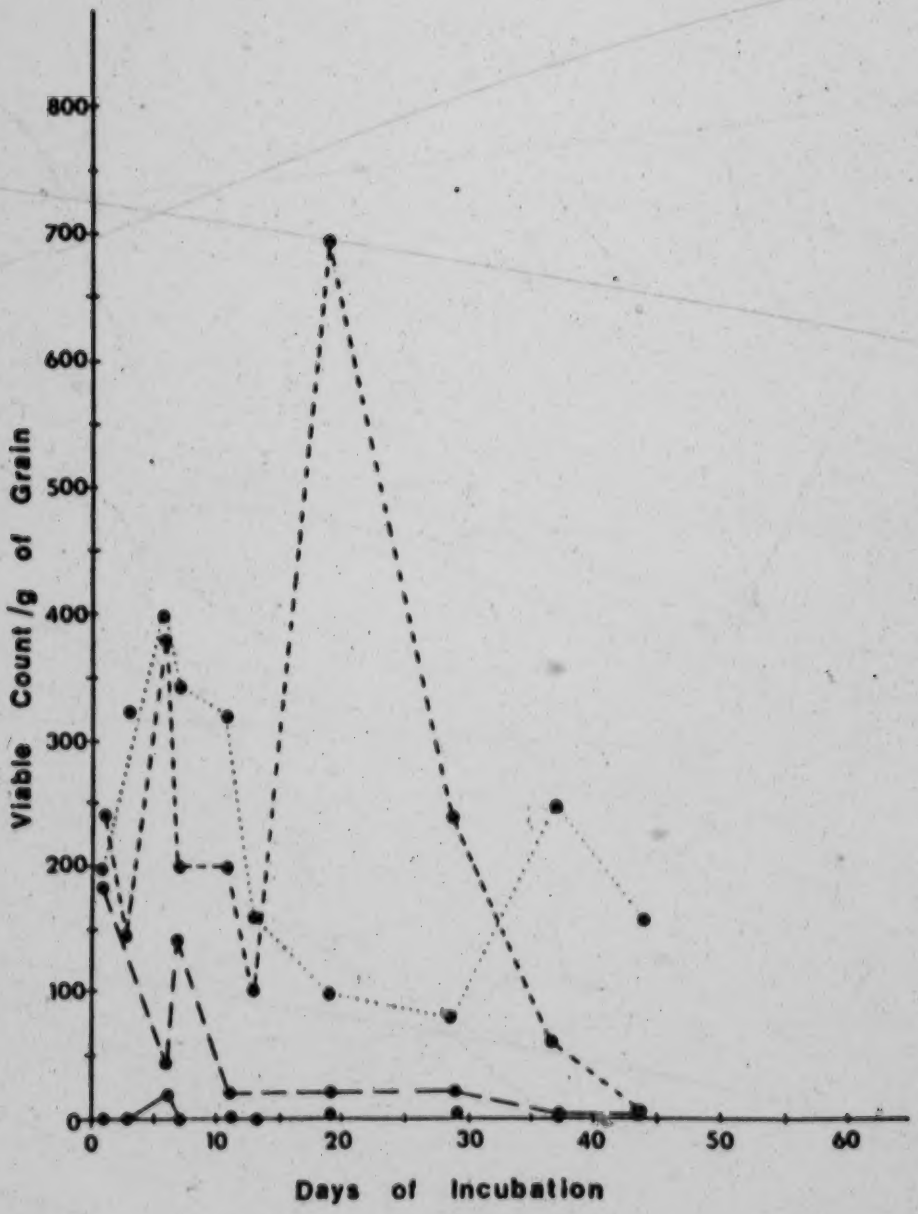
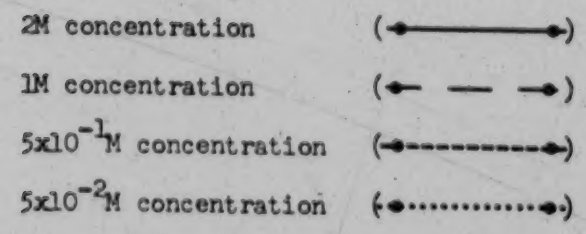
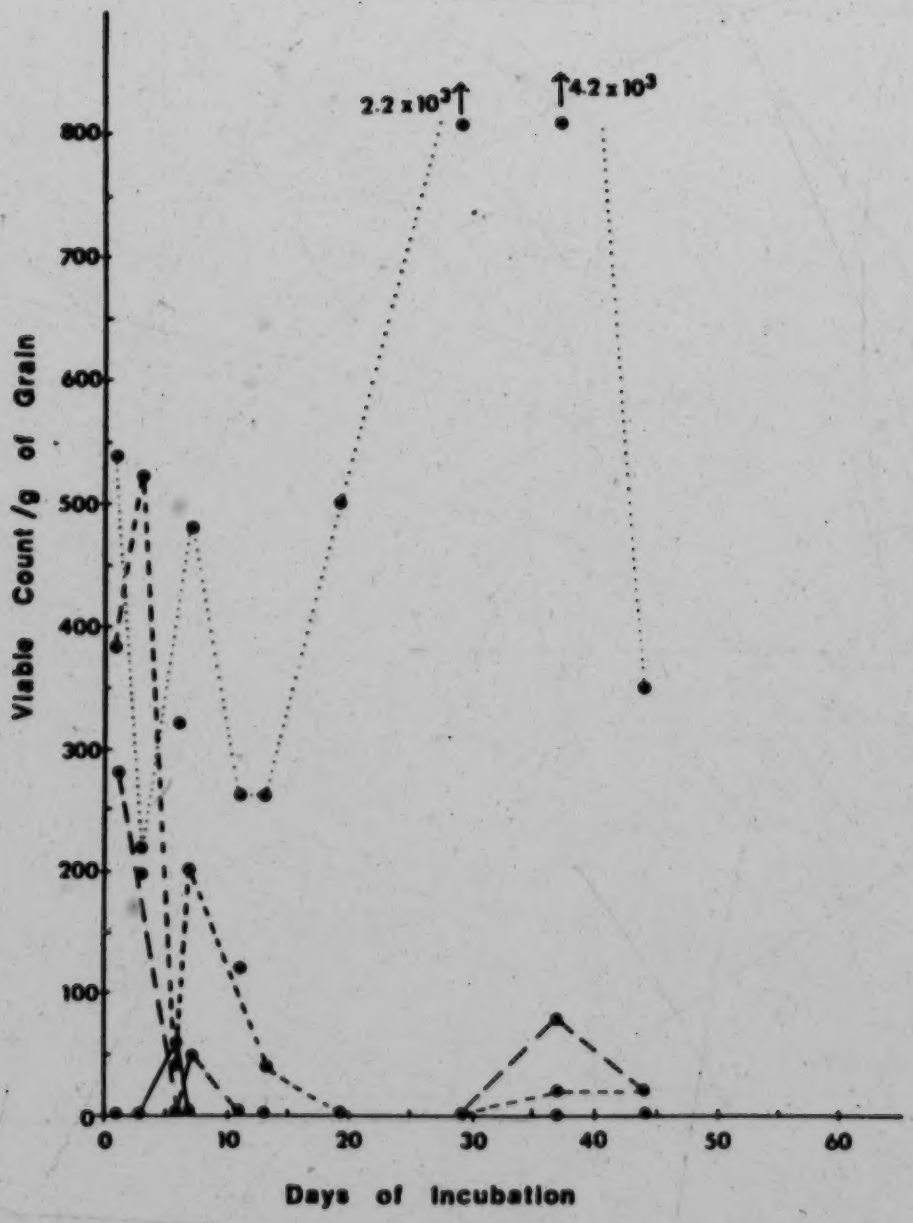

Viable Count /g of Grain
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Days of Incubation

FIGURE 12

GROWTH RESPONSE TO HIGHER CONCENTRATIONS OF PROPIONIC ACID AT INCREASING PERIODS OF INCUBATION

Ten g of untreated grain (viable count = 8.2×10^7) were added to 90 ml of the appropriate concentration of acid and after mixing, incubated at 30°C. At the indicated times, 0.1 ml samples of the supernatants were analysed for viable organisms.




2.2 x 10³
4.2 x 10³
800
700
600
500
400
300
200
100
0
Viable Count /g of Grain
0
10
20
30
40
50
60
Days of Incubation

for the grain microflora after prolonged exposure would no doubt be of a handling advantage. This could be applicable to the 1M concentrations of acid tested. At this concentration, acetic acid was biocidal after 36 days and propionic acid as early as 6 days. As for the $5x10^{-1}$M concentration of each of the two acids, results are not as clear. A distinct logarithmic decrease in the number of survivors at these two concentrations of acid was not observed. The lowest concentration of propionic and acetic acid, namely $5x10^{-2}$M, showed no evidence of biocidal activity at any time and may have even supported the growth of some remaining microbial populations.

VII. Relationship Between Grain Surface pH and Total Viable Count of Grain Microflora

The pH of grain is attributed to many interdependent factors including pH of microbial metabolic products, pH of soil and harvesting environment as well as the buffering action of proteins and other grain constituents (Anderson and Alcock, 1954). A study was undertaken to determine the relationship between the pH of an aqueous grain extract and the total viable count of microorganisms on untreated grain. A parabolic representation was implicated as viable counts have a tendency to decline at extreme pH values, Figure 13. Extreme pH values did exhibit a significantly lower number of grain microorganisms.

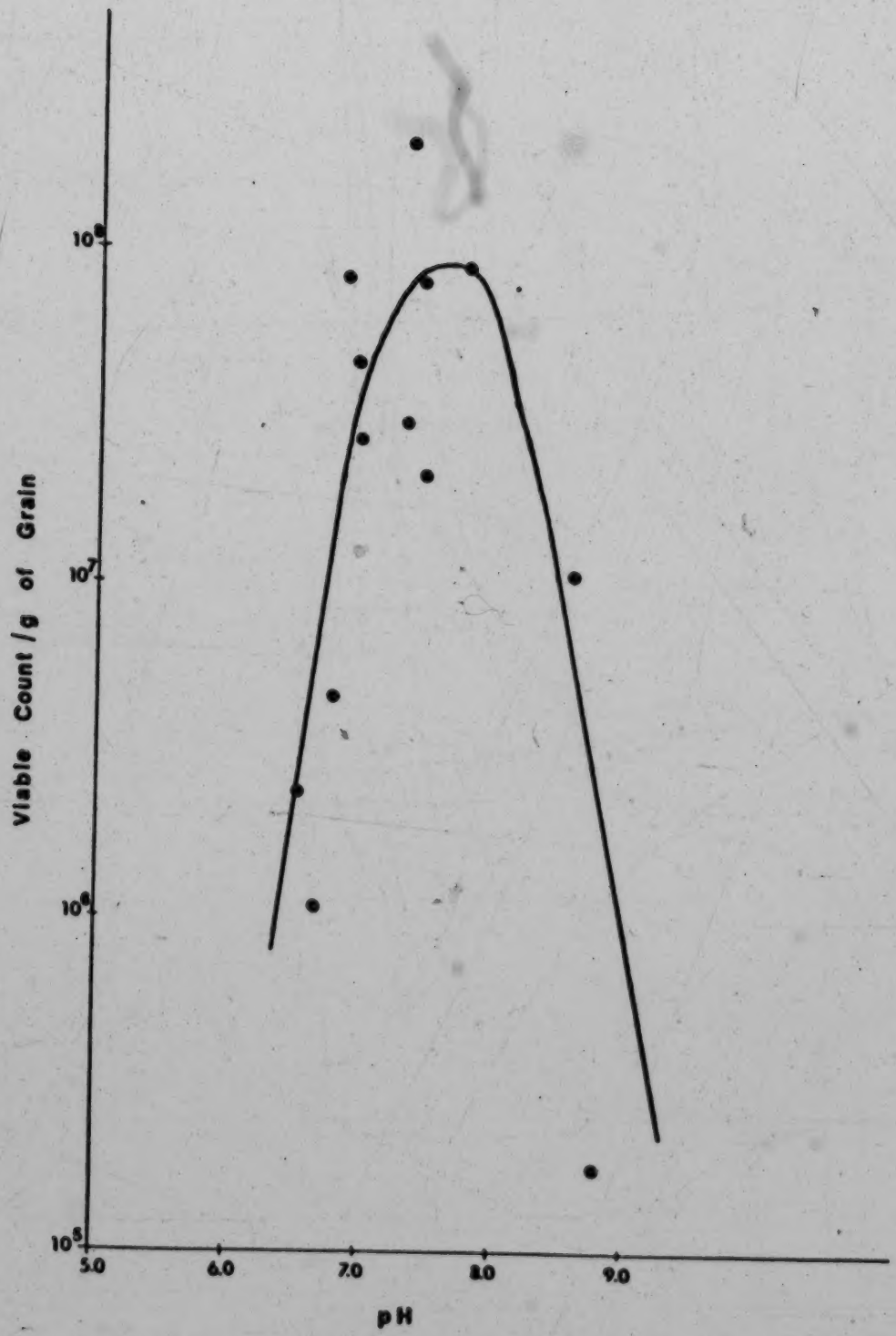
VIII. Evidence of Preservative Acid Utilization by the Grain Microflora

An investigation was conducted to determine the possibility of the organic acid preservatives being used as a source of energy by the grain microflora. For this purpose, labelled carbons of carboxylic

FIGURE 13

RELATIONSHIP BETWEEN GRAIN SURFACE pH AND TOTAL VIABLE COUNT

The pH of 5 g of saturated grain was determined. Ten g of each grain sample was added to 90 ml of water and after dispersion, 0.1 ml samples of the appropriate dilution analysed for viable organisms.


Viable Count /g of Grain
10^8
10^7
10^6
10^5
5.0
6.0
7.0
8.0
9.0
pH

groups of acetic acid and sodium propionate were introduced into the reaction mixtures with the intent of trapping any respired $^{14}CO_2$ in phenethylamine.

Initial preparations involved verification of the radiochemical purity of the radioactive working solutions by ascending paper chromatography, and the design of a reaction vessel which would serve in the radiorespirometric studies. The compositions of the reaction mixture in each of four reaction vessels which comprised a single run and only one concentration of one of the labelled acids at a time is illustrated in Table X. Preparative procedures required of any of the individual constituents of these reaction mixtures have already been described in Materials and Methods IX.

First the apparatus was autoclaved, then sterile grain was added aseptically, followed by inoculum and Bacto - Tryptone water. Upon the addition of the labelled acid solutions, sampling commenced and was carried out as described in Materials and Methods X.

It was expected that grain microflora would utilize the acids at low concentrations since the organisms were mainly heterotrophs. However it was of interest to compare this predicted rate with that at a higher acid concentration : results for a 0.001M solution of labelled acetic and propionic acids are presented in Figures 14 and 15. The total amount of ^{14}C trapped in phenethylamine at the designated times is expressed in nmol acid equivalents/ml of reaction mixture as derived from the following expression:

$$\frac{\text{dpm } ^{14}C \text{ evolved}}{\text{45 ml reaction mixture}} \div \frac{\text{initial dpm}}{\text{nmol acid in 45 ml reaction mixture}}$$

TABLE X

COMPOSITION OF REACTION MIXTURES IN VESSELS FOR $^{14}CO_2$ PRODUCTION STUDIES

Composition		Vessel Number 1	2	3	4
Sterile grain	5 g	*A	A	**N/A	N/A
Inoculum (total viable count = 10^9 cells/ml)	43 ml	A	N/A	N/A	A
Sterile distilled water . . .	43 ml	N/A	A	A	N/A
Sterile 2.3% Bacto - Tryptone water	1 ml	A	A	A	A
0.045M or 0.90M acetic or propionic acid (radioactive concentration = 1 µc/ml) . .	1 ml	A	A	A	A

Vessels 2 and 3 serve as controls for 1 and 4, respectively.

Whether the presence of grain has any effect on the amount of $^{14}CO_2$ produced by the grain microflora, is the basis for the constituents comprising the reaction mixtures of vessels 1 and 4.

*A, applicable, denotes that the particular component is added to the reaction mixture.

**N/A, not applicable, denotes that the particular component is not added to the reaction mixture.

FIGURE 14

INCREASED PRODUCTION OF VOLATILE ACIDIC ^{14}C FROM A DILUTE SOLUTION OF LABELLED ACETIC ACID CONTAINING GRAIN MICROORGANISMS

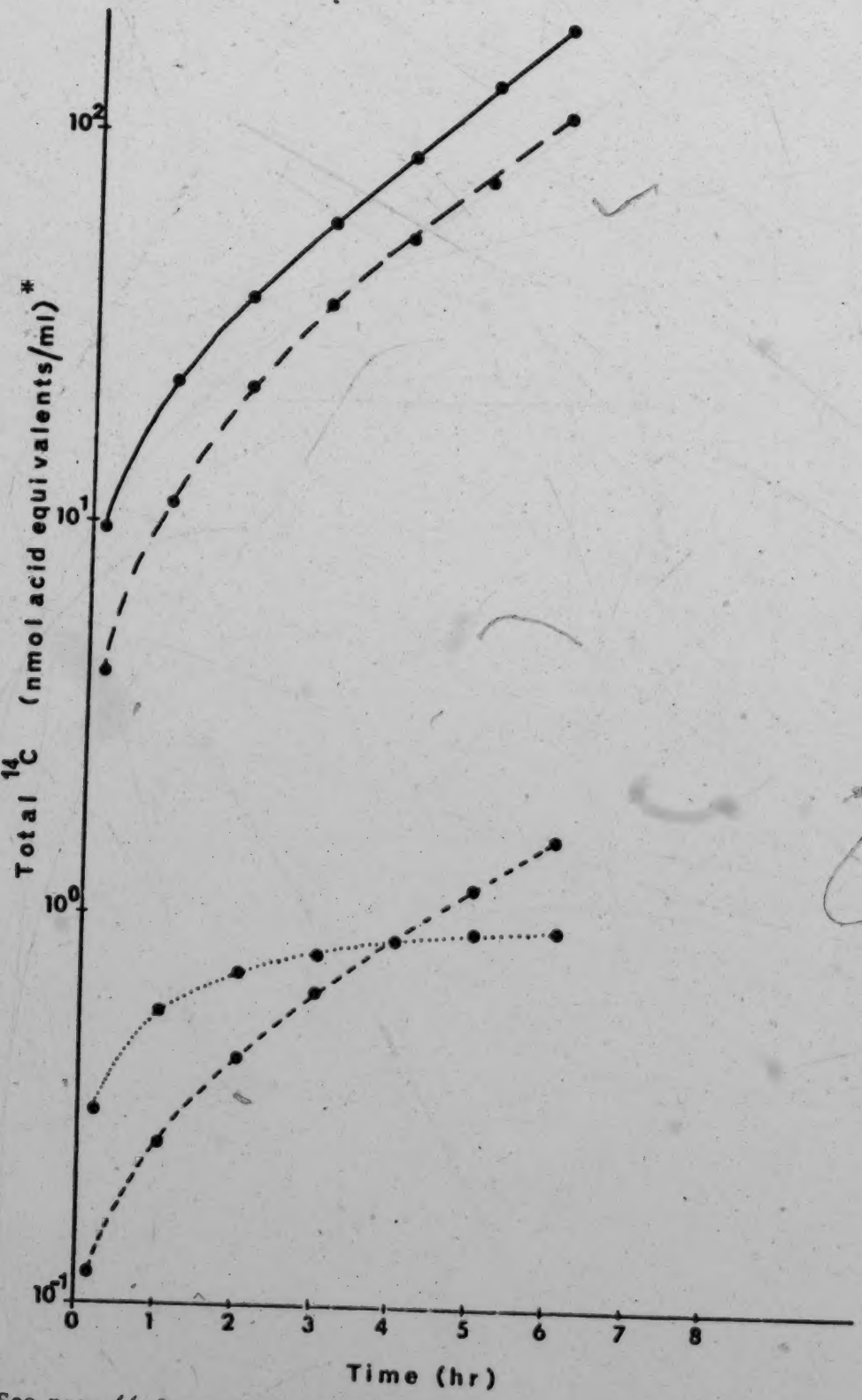
Forty-three ml of a suspension of grain microorganisms in 0.05% Bacto - Tryptone water containing $1x10^9$ cells/ml were incubated with 1 ml of 2.3% Bacto - Tryptone water and 1 ml of ^{14}C acetic acid (.045M.) in the presence and absence of 5 g of sterilized grain. A grain suspension and a solution containing only ^{14}C acetic acid were used as controls. The evolution of ^{14}C was measured as outlined in the Methods.

Grain + inoculum

Grain + no inoculum

Inoculum + no grain

No inoculum + no grain



*See page 66 for method of calculation.

FIGURE 15

INCREASED PRODUCTION OF VOLATILE ACIDIC ^{14}C FROM A DILUTE SOLUTION OF LABELLED PROPIONIC ACID CONTAINING GRAIN MICROORGANISMS

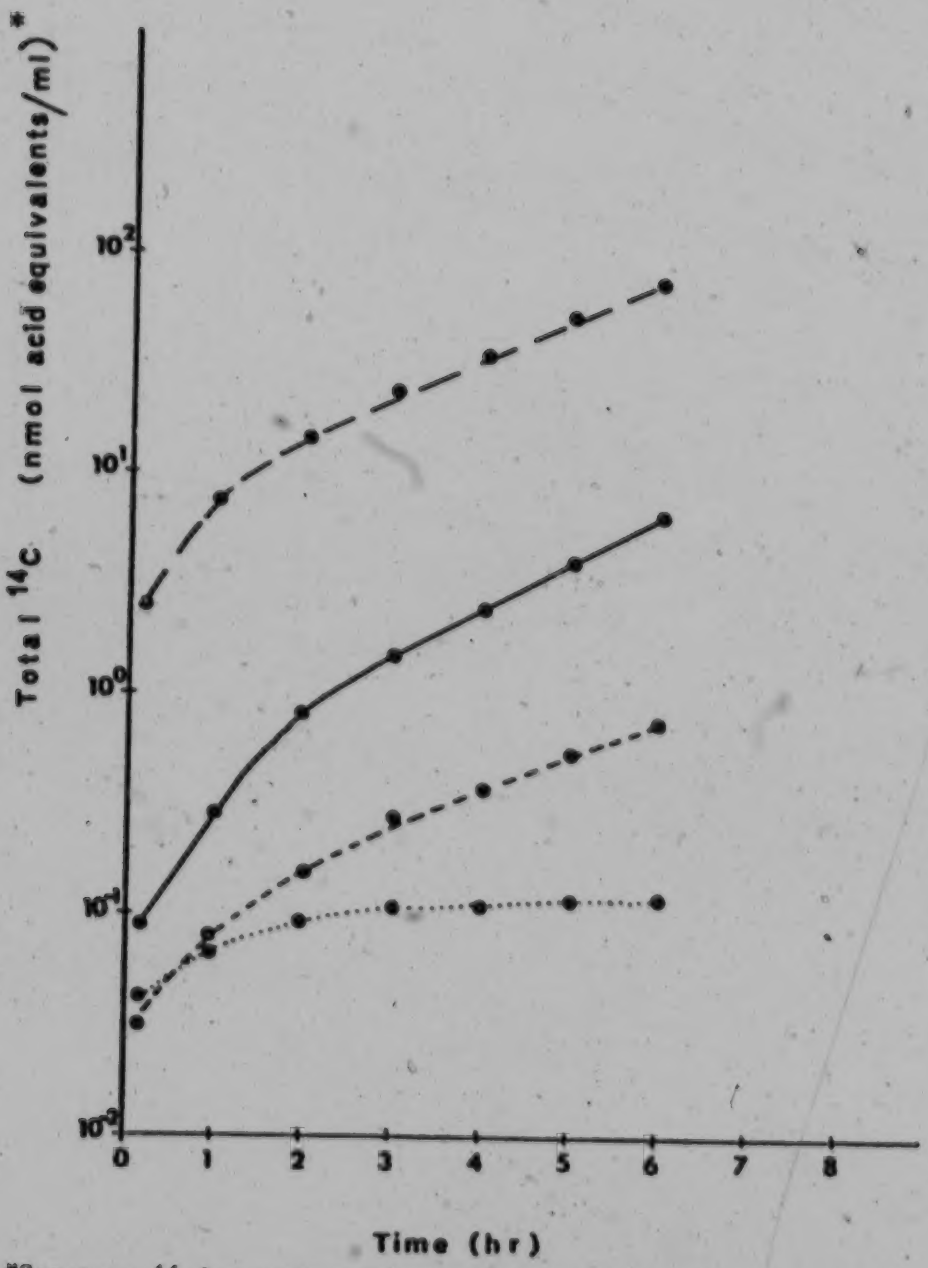
Forty-three ml of a suspension of grain microorganisms in 0.05% Bacto - Tryptone water containing $1x10^9$ cells/ml were incubated with 1 ml of 2.3% Bacto - Tryptone water and 1 ml of ^{14}C propionic acid (.045M) in the presence and absence of 5 g of sterilized grain. A grain suspension and a solution containing only ^{14}C propionic acid were used as controls. The evolution of ^{14}C was measured as outlined in the Methods.

Grain + inoculum (—●———●—)

Grain + no inoculum (-●--------●-)

Inoculum + no grain (—● — —●—)

No inoculum + no grain (●.....●.)



*See page 66 for method of calculation.

When microbial cells were present, the quantity of radioactive carbon evolved is expressed in nmol acid equivalents/10^9 cells. Since viable counts were not always 10^9 cells/ml, this was corrected for in the following way:

$$\frac{(\text{nmol acid equivalents/ml})\ (10^9)}{\text{viable count/ml inoculum}}.$$

In the presence of inoculum, with or without sterile grain, more ^{14}C was detected as compared to the controls. These controls which were lacking in inoculum, showed the amount of radioactivity lost as a result of the volatility of the acids and the ability of the grain alone to metabolize the acids. The addition of grain did reduce the amount of ^{14}C evolved from reaction mixtures containing microbial cells. This could imply that the grain was absorbing labelled acid thus minimizing its availability or, perhaps, excreting some substance which hindered utilization of the acid by the grain microflora. The grain microflora utilized acetic acid more readily than propionic, suggesting that a smaller segment of the grain microflora population was attributed with the faculty of utilizing propionic acid.

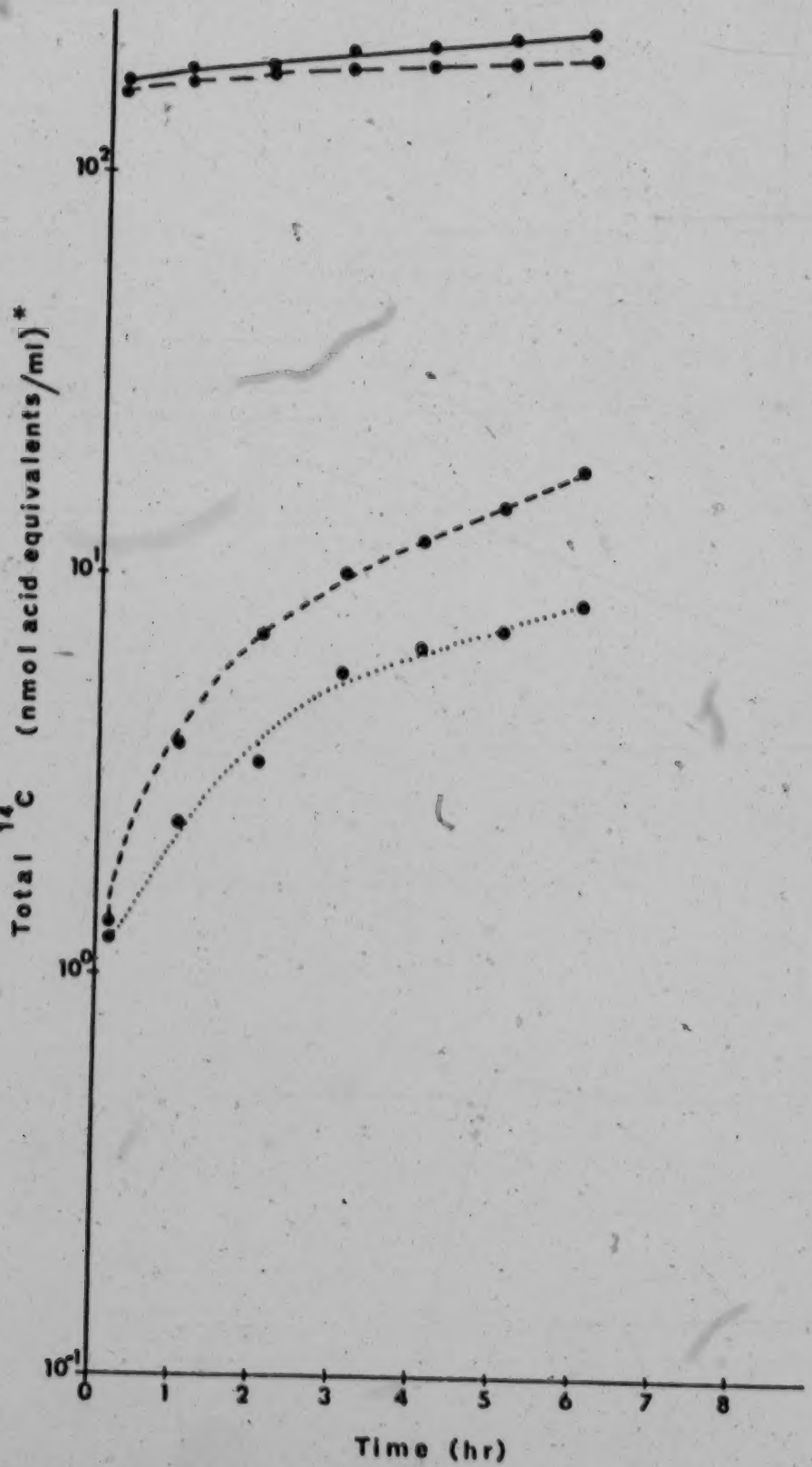
When the concentration of acid was increased to 0.02M, Figures 16 and 17, oxidation of acetic acid by the grain microflora was still evident since the amount of ^{14}C released in the presence of inoculum was higher than that of the controls. But for propionic acid, results approximated those of the controls. Thus at a 0.02M concentration of propionic acid, the grain microflora displayed no ability to utilize

FIGURE 16

INCREASED PRODUCTION OF VOLATILE ACIDIC ^{14}C
FROM A CONCENTRATED SOLUTION OF LABELLED ACETIC ACID
CONTAINING GRAIN MICROORGANISMS

Forty-three ml of a suspension of grain microorganisms in 0.05% Bacto - Tryptone water containing $1x10^9$ cells/ml were incubated with 1 ml of 2.3% Bacto - Tryptone water and 1 ml of ^{14}C acetic acid (0.9CM) in the presence and absence of 5 g of sterilized grain. A grain suspension and a solution containing only ^{14}C acetic acid were used as controls. The evolution of ^{14}C was measured as outlined in the Methods.

Grain + inoculum (●——●)
Grain + no inoculum (●-------●)
Inoculum + no grain (● — ●)
No inoculum + no grain (··●·····●·)

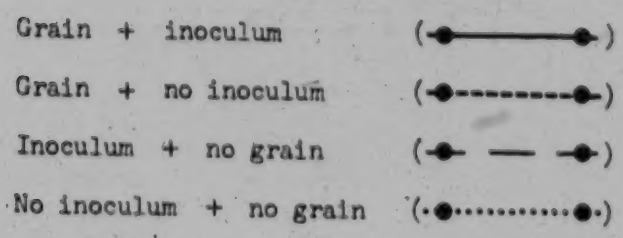


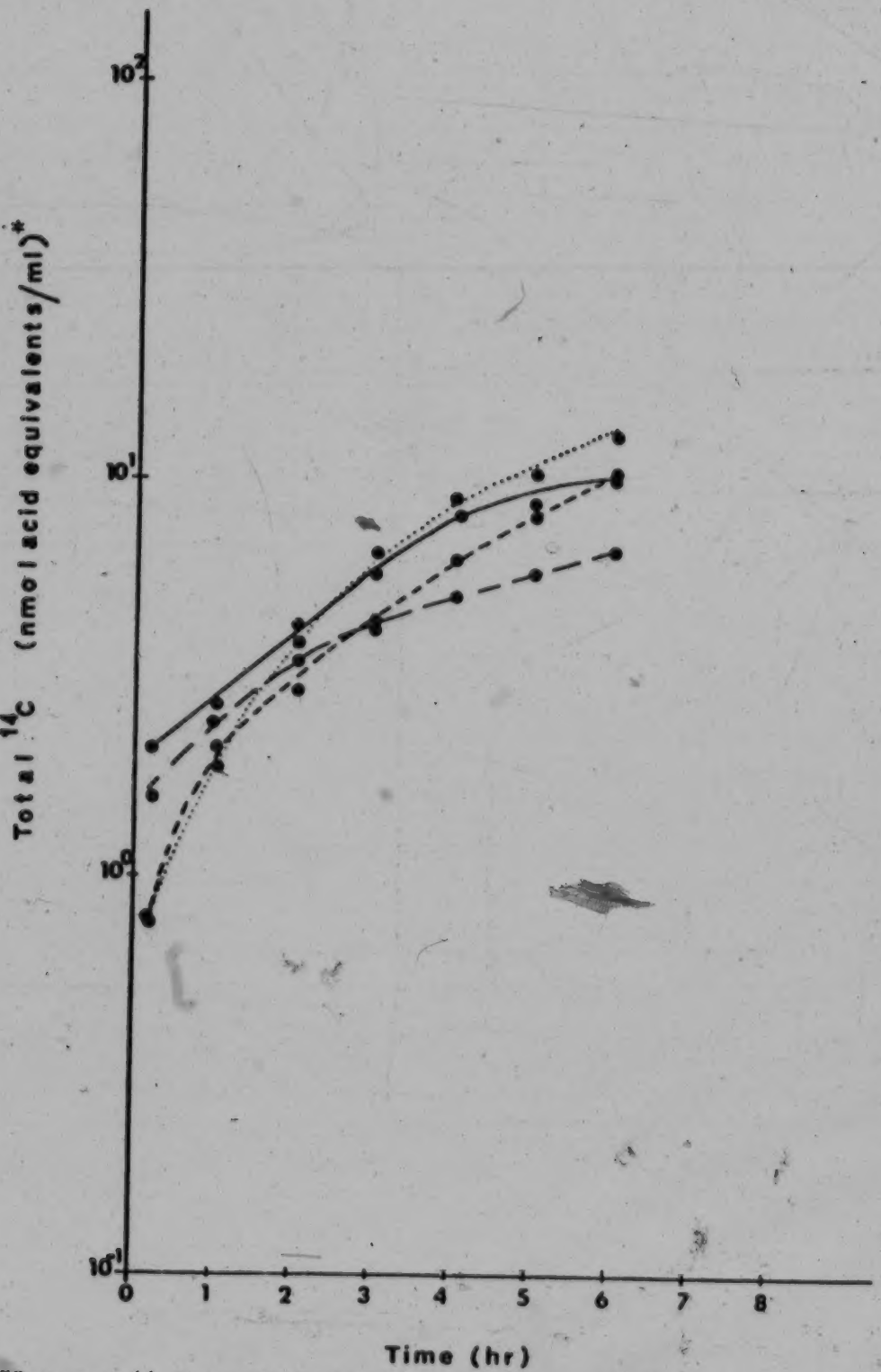
*See page 66 for method of calculation.

FIGURE 17

INCREASED PRODUCTION OF VOLATILE ACIDIC ^{14}C
FROM A CONCENTRATED SOLUTION OF LABELLED PROPIONIC ACID
CONTAINING GRAIN MICROORGANISMS

Forty-three ml of a suspension of grain microorganisms in 0.05% Bacto - Tryptone water containing $1x10^9$ cells/ml were incubated with 1 ml of 2.3% Bacto - Tryptone water and 1 ml of ^{14}C propionic acid (0.90M) in the presence and absence of 5 g of sterilized grain. A grain suspension and a solution containing only ^{14}C propionic acid were used as controls. The evolution of ^{14}C was measured as outlined in the Methods.





*See page 66 for method of calculation.

the acid during an exposure period of 6 hr. Several steps were performed upon termination of the experiment to examine the distribution of radioactivity amongst the components of the reaction mixture with the ultimate aim of accounting for all the radioactivity put into the system. A summary of all findings is presented in Table XI.

The percentage of $^{14}CO_2$ released after 6 or 24 hr was obtained by subtracting the amount of ^{14}C lost due to the volatility of the acid from the nmol of ^{14}C evolved/10^9 cells at these times, dividing by the initial number of nmol/10^9 cells present and multiplying by 100%. For 0.001M concentrations with and without grain, oxidation was still prevalent after 6 hr since the percentage of $^{14}CO_2$ at 24 hr was greater than at 6 hr. For 0.02M acetic acid, there was a negligible increase after initial exposure. Perhaps the acetic acid utilizing population was unable to cope with the sudden increase and was killed as suggested by the significant decrease in viable count after 24 hr. No $^{14}CO_2$ was detected with 0.02M propionic acid even after a lag period of 24 hr. The degree of reduction in total viable microorganisms suggested that this concentration of acid was probably biocidal for the propionic utilizing segment of the grain microflora. An insignificant change from the initial viable count does not necessarily imply that a particular concentration of acid was not biocidal to some grain microorganisms as a loss in number could have easily been compensated for by an actively growing population such as the acetic and/or propionic acid utilizing microorganisms.

TABLE XI

SUMMARY OF RADIOACTIVE DATA

Criterion	Acetic Acid - 1 - ^{14}C				Propionic Acid - 1 - ^{14}C			
	Inoculum + Grain		Inoculum - Grain		Inoculum + Grain		Inoculum - Grain	
	0.001M	0.02M	0.001M	0.02M	0.001M	0.02M	0.001M	0.02M
Initial rate (nmol $^{14}CO_2$/hr/10^9cells)	6.51	11.20	13.22	11.20	0.14	0	5.26	0
% $^{14}CO_2$ released after 6 hr	9.19	0.37	14.91	0.34	0.72	0	9.72	0
% $^{14}CO_2$ released after 24 hr	26.84	0.40	26.90	0.34	15.90	0	20.07	0
% radioactivity in cells (after 24 hr)	27.78	0.62	30.90	0.81	9.19	0.28	24.61	0.18
% radioactivity in medium (after 24 hr)	35.58	100.00	1.99	104.00	63.28	85.00	3.38	96.42
% radioactivity in grain (after 24 hr)	1.77	8.24	* —	* —	1.54	9.36	* —	* —

TABLE XI (Continued)

Criterion	Acetic Acid - 1 - ^{14}C				Propionic Acid - 1 - ^{14}C			
	Inoculum + Grain		Inoculum - Grain		Inoculum + Grain		Inoculum - Grain	
	0.001M	0.02M	0.001M	0.02M	0.001M	0.02M	0.001M	0.02M
Total % radioactivity accounted for	91.97	109.26	59.79	105.15	89.91	96.64	48.06	96.60
Viable count/ml (initial)	8.5×10^8	3.6×10^8	8.5×10^8	3.6×10^8	1.4×10^9	1.8×10^9	1.4×10^9	1.8×10^9
Viable count/ml (after 24 hr)	6.7×10^8	2.5×10^7	9.2×10^8	5.3×10^7	4.6×10^8	1.2×10^7	1.2×10^9	4.4×10^7

*No grain present.

Even after 24 hr, the grain microflora still oxidized more acetic acid than propionic acid.

The following expression was used to estimate the percentage of radioactivity in the grain microflora:

$$\frac{\text{(dpm in cells/45 ml reaction mixture) (100\%)}}{\text{(dpm initially present) - (dpm lost from controls after 24 hr)}}$$

This is further proof that the cells do indeed take up the labelled acid and oxidize it.

The percentage of radioactivity not present in the cells after 24 hr was calculated in the following manner:

$$\frac{\text{(dpm not in cells/45 ml reaction mixture) (100\%)}}{\text{(dpm initially present) - (dpm lost from controls after 24 hr)}}$$

Grain definitely absorbed labelled acid and the more acid present, the greater the absorption. Percentage of label taken up by the grain was obtained thus:

$$\frac{\text{(dpm of grain mash filtrate + dpm of grain mash) (100\%)}}{\text{(dpm initially present) - (dpm lost from controls after 24 hr)}}$$

By adding the results of the last four criteria discussed, the total percentage of radioactivity accounted for is determined. Fifty percent of the radioactivity for 0.001M acetic and propionic acid where only inoculum is present and no grain, cannot be traced. The labelled carbon may have been incorporated into some molecule other than CO_2.

IX. Examination of Survivors after Treating Grain with Various Concentrations of Acid

The microorganisms capable of surviving after treatment of grain with various concentrations of acid were categorized empirically by morphology and colonial characteristics.

a. Gram-positive or Gram-variable rods occurring singly or in chains producing endospores when grown aerobically on PCA were classified as "Bacillus".

b. Unicellular, nonsporing Gram-variable cocci occurring singly, in pairs or in irregular clusters were assumed to belong to the genus "Micrococcus".

c. Gram-negative straight, nonsporing rods producing deep yellow or orange opaque colonies, usually entire but sometimes showing flexing mobility were assumed to be "Flavobacteria" or "Cytophaga".

d. "Arthrobacter" were recognized by observing a change in morphology in broth grown cultures. Cells 2-3 hr old are typically pleomorphic rods, usually Gram-positive. Cells 12-24 hr old are oval or coccoid in shape, usually Gram-negative.

e. Members of the "Enterobacteriaceae" were detected by their Gram reaction and ability of cells from single colonies to produce acid and gas or acid alone from lactose in conventional lactose broth.

Although bacteria mentioned are those that can be roughly characterized by head-mark, many of the bacteria found after acid treatment could not be categorized by the limited number of tests used.

Upon the addition of a dilute concentration of acetic or propionic acid, namely $1x10^{-4}$M, all bacterial colonies described above were found. At a $1x10^{-3}$M concentration of either acid, colonies characteristic of "Bacillus", "Micrococcus" and "Enterobacters" dominated. Increasing the concentration from $5x10^{-3}$M to $1x10^{-1}$M, a pH range of approximately 2.8 to 4.0, selected for molds and yeasts. Five types of molds and two yeasts were recovered, but no attempt was made at further classification. In addition, rod-shaped Gram-negative microorganisms producing red opaque colonies and acid in lactose broth appeared at a $1x10^{-1}$M concentration of acetic acid. These were assigned to the genus "Serratia". Highly concentrated solutions of acetic and propionic acids, that is, 1M and 1.5M, eliminated all Gram-negative rods except for "Enterobacters". Gram-positive sporing rods also survived and were most prevalent. Spores of "Bacillus" would be more resistant to killing, thus demanding such a high biocidal concentration of acid as 2M.

Studies involving preservatives and disinfectants are routinely conducted with pure cultures of microorganisms, mainly pathogens or "nuisance" microorganisms. Since the grain microflora consists of a wide array of microorganisms all having the potential

of exploiting the nutrients supplied by the grain and thereby causing spoilage, it was more desirable to examine effects on the total grain microflora rather than a pure culture of isolated grain microorganisms. Studies with pure cultures of each of the most prevalent microorganisms would show individual effects, as different microorganisms do not respond equally to the same concentration of acid and low concentrations may even stimulate growth. Unless such investigations are carried out, it is impossible to determine whether a concentration of acid exhibiting a decrease in viable count is bacteriostatic, or supports the growth of some microorganisms. The only conclusion that can be made is that it is biocidal for at least some microorganisms.

SUMMARY AND CONCLUSIONS

A simple but very reliable test was designed to determine the sterilizing potential of grain preservatives on independent grain samples. The advantage of its simplicity is that it can be performed by farm personnel who have been exposed to a minimum of scientific knowledge.

The two weak organic acids, namely, acetic and propionic, are the basic constituents of the particular moist grain preservative under microbiological investigation. Under the defined experimental conditions, exposure of the grain microflora to increasing concentrations of each of these acids displayed a geometric decline in the number of survivors. Moreover, both acids had equivalent killing abilities. The presence of both exhibits no synergism in the killing of the grain microflora. The implication involved here is an important one as these acids, together, are a by-product of an industrial chemical process and their separation would be a costly venture for the company disposing of them if their individual killing effects were significantly greater than when combined.

Some property of these weak organic acids other than pH is responsible for the preservative action. All data obtained strongly implicate that it is the concentration of undissociated acid.

Because of easier handling, salts of weak organic acids are often used in the preservation of foodstuffs instead of the acids, themselves, which are very corrosive and volatile. Studies carried out showed that the sodium salts of the preservative acids under

investigation were not as effective in sterilizing grain as the acids. Only high concentrations of these weak organic acids with characteristic low pH readings were biocidal for the grain microflora. On the other hand, a buffer consisting of the acid and its sodium salt, of higher pH whose concentration of undissociated acid was equivalent to that of the biocidal concentration of acid, was also found to be biocidal. It is therefore recommended that such less corrosive solutions be tested under field conditions for their ability to preserve moist grain.

Experiments conducted show that bacteriostatic concentrations of the preservative acids may become biocidal for the grain microflora after prolonged exposure. Biocidal concentrations maintain their control indefinitely and manifest the majority of their killing within one minute of contact to the existing grain microflora.

A parabolic relationship was implicated between grain surface pH and total viable count of the grain microflora. Grains with extreme grain surface pH values exhibited lower total viable counts. This could be significant in the amount of acid necessary for their preservation.

Data from radiorespirometric experiments confirmed the existence of microbial populations among the grain microflora capable of utilizing dilute concentrations of the preservative acids. This is not an unusual feature considering that the majority of grain micro-organisms are heterotrophs. Acetic acid was oxidized more readily than propionic acid. At a higher concentration, no propionic acid and only a small percentage of acetic acid breakdown was detected. The preservative acids are absorbed by the grain with no preference for one

acid or the other and larger concentrations result in more acid absorption by the grain. Thus, the amount of acetic acid comprising the preservative mixture must not be of a very low concentration. If moist grain is treated with a concentration of acid preservative such that it is biocidal for the grain microflora and protected in a manner so as to prevent maximum volatilization of the acids and the introduction of microorganisms during storage, there is no reason for grain spoilage to occur.

The profuse presence of sporing and capsular microorganisms constituting the grain microflora could be the basis for the need of using such a high concentration of acid as 2M to sterilize grain.

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APPENDIX I

COMMENTARY ON STATISTICS EMPLOYED

Once results are secured from investigations involving samples from populations, statistical decisions concerning these populations are often called for. Prior to experimentation, the common course is to make a guess, or more correctly, a hypothesis. The type of statistical hypothesis formulated is called a null hypothesis which states that there is no difference between the elements in question and any observed difference is due to chance alone. The statistical procedures applied in accepting or rejecting this null hypothesis are referred to as tests of significance.

The possibility of making errors in accepting or nullifying the null hypothesis cannot be disregarded. To compensate for this, levels of significance are also chosen before any samples are selected for experimentation. They indicate the maximum probability of rejecting a null hypothesis when it actually should have been accepted. The most common levels of significance are .05 and .01. For a .05 level of significance, this means that there are 5 chances out of 100 in making the above error or, similarily, that the right decision was made with 95% confidence.

All statistics applied to the data presented in the Results and Discussion section were based on a normally distributed population.

The "Student's" two-tailed t-statistic is used to test the null hypothesis that the means of two samples of a certain size

(usually not greater than thirty) are equal, assuming that they are drawn from normal populations whose standard deviations are equal. The two-tailed implies that extreme values of the t-distribution, that is, values in both tails of the distribution are taken into consideration.

The analysis of variance is an extension of the "Student's" t-test but it is more precise in that the total sum of squares is partitioned into components which are obvious sources of variation such as that within treatments or between treatments. It allows for comparing more treatments with fewer replications. In an analysis of variance for a completely randomized design, the number of replicates for each of the treatments need not be equal. If unequal, a new computation for the correction term is employed. From the null hypothesis stating that the samples are all from the same population, it is assumed that the calculated variances from the sources of variation are all estimates of the same true variance. The F-statistic is introduced and from the F-distribution the probability that a given value of F will be exceeded through chance alone can be determined.

If, after an analysis of variance it was found that all samples were not from the same population, the Duncan's new multiple-range test is a simple way of resolving which of the treatments are significantly different from each other and which are not.

The split-plot design is used for factorial experiments. Whole units consisting of levels of one or more factors are divided into subunits to which levels of other factors are applied. It increases the scope of the experiment by breaking down the analysis of

variance between different factors and is desirable when greater precision for comparing certain factors is required than for others.

The relationship between two variables or effects of different factors is often expressed graphically. Initially, points are plotted on a rectangular coordinate system to obtain a scatter diagram after which an appropriate curve is visualized. If represented by a straight line, a linear relationship between the variables is deduced and if not linear, a non-linear relationship. Non-linear relationships are frequently reduced to linear relationships by appropriate transformation of the variables, and another scatter diagram is secured of the transformed variables. Often log-log or semi-log graph paper is used for this purpose. Curve fitting involves finding the correct mathematical equation for the curves which fit a given set of data be they those of a parabola, exponential, or a geometric curve. Drawing a curve free hand is subject to error and the least square method which involves solving the normal equations for a least square parabola, exponential or geometric curve or otherwise, is utilized to obtain the best fitting curve.

The computational procedures for the various statistics employed were procured from the following sources: Steel and Torrie's text entitled Principles and Procedures of Statistics (1960) and a review article by Eisenhart and Wilson (1943-1944).

APPENDIX II

A REVIEW OF THE CHEMISTRY INVOLVED IN STUDYING THE PRESERVATIVE ACIDS

Acetic acid, CH_3COOH, and propionic acid, CH_3CH_2COOH, are weak organic acids and therefore do not ionize to any appreciable extent when in solution. The degree to which they are dissociated is given by the Ka expression which is simply a mathematical equation stating the equilibrium that exists between the ionized and unionized forms:

$$Ka = \frac{[H^+]\ [A^-]}{[HA]},$$

where Ka is the equilibrium constant for the dissociation of the acid, $[H^+]$ the concentration of hydrogen ions from which pH is derived, $[A^-]$ the concentration of ionized acid and $[HA]$ the concentration of unionized acid.

Utilizing the Ka expression, the pH of an organic acid solution of a known molarity can be determined:

$$[H^+] = [A^-] = \frac{-Ka + \sqrt{(Ka)^2 - 4(Ka)(M)}}{2}$$

The donation of hydrogen ions from the ionization of water molecules is insignificant since weak acids are obviously much stronger acids than water. Once the concentration of hydrogen ions has been calculated, the concentration of the undissociated acid is found by subtracting $[H^+]$ from the initial acid concentration.

When a weak acid and its salt are combined, the salt of the weak acid suppresses the dissociation of the acid since it enters into equilibrium with the acid, hydrogen and dissociated acid ions. The desired amounts of weak acid and the salt of the weak acid required to make up an acid-salt solution of a known molarity and pH can be obtained from the Henderson - Hasselbalch equation:

$$pH = pKa + \log_{10} \frac{[A^-]}{[HA]}$$

or the Ka expression where $[A^-]$ will denote the concentration of salt and $[HA]$ the amount of acid required.

A reliable reference for computational procedures is the book entitled Biochemical Calculations by Segel (1968).